Award Number: **W81XWH-11-1-0105**

TITLE: **A Drug Discovery Partnership for Personalized Breast Cancer Therapy**

PRINCIPAL INVESTIGATOR: **Maryam Foroozesh, Ph.D.**

CONTRACTING ORGANIZATION: **Xavier University of Louisiana**
New Orleans, LA 70125

REPORT DATE: **September 2015**

TYPE OF REPORT: **Annual**

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release; Distribution Unlimited

REPORT DOCUMENTATION PAGE		*Form Approved* *OMB No. 0704-0188*
1. REPORT DATE September 2015	**2. REPORT TYPE** Annual	**3. DATES COVERED** 09/01/14- 08/31/15
4. TITLE AND SUBTITLE A Drug Discovery Partnership for Personalized Breast Cancer Therapy		**5a. CONTRACT NUMBER** W81XWH-11-1-0105 **5b. GRANT NUMBER** BC102922 **5c. PROGRAM ELEMENT NUMBER**
6. AUTHOR(S) Maryam Foroozesh (PI), Thomas Wiese, Jayalakshmi Sridhar, Barbara Beckman, Matthew Burow, Frank Jones, Cheryl Stevens E-Mail: mforooze@xula.edu		**5d. PROJECT NUMBER** **5e. TASK NUMBER** **5f. WORK UNIT NUMBER**
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Xavier University of Louisiana New Orleans, LA 70125		**8. PERFORMING ORGANIZATION REPORT**
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		**10. SPONSOR/MONITOR'S ACRONYM(S)** **11.SPONSOR/MONITOR'S REPORT NUMBER(S)**

12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT Xavier University of Louisiana is in the unique position of developing capability in drug discovery especially in the areas of cancer and health disparities. A significant proportion of the funded research on Xavier's campus including collaborative projects involving Tulane University are related to cancer, drug design, synthesis, and drug delivery. This project **expands the partnership between Xavier University and Tulane Cancer Center to develop and validate drugs for breast cancer therapy.** The Drug Design Team at Xavier consists of experts in computer-aided drug design methods and synthesis and has formed a productive partnership with the Cancer Drug Validation Team at the Tulane Cancer Center. This inter-university collaboration involves training Xavier researchers, including undergraduate students, to carryout the experiments necessary to determine if new compounds would be suitable as new drugs to treat breast cancer. Three separate studies are ongoing as subprojects: (1) Design and synthesis of novel ceramide analogs that potentially reverse chemotherapy drug resistance, (2) Design and synthesis of small molecule inhibitors of HER2 tyrosine kinase to suppress tumorigenesis, and (3) Identification of compounds with the potential for estrogen receptor activity.

15. SUBJECT TERMS Breast cancer, Partnership, Training

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT UU**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE** PERSON USAMRMC
a. REPORT U	**b. ABSTRACT** U	**c. THIS PAGE** U	UU	72	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Page

INTRODUCTION:

This project brings together the skills and resources of Xavier University and Tulane University researchers to form a collaborative team in the areas of drug design and validation. This inter-university collaboration whereby a number of *in vitro* and *in vivo* approaches are applied for validation of lead compounds designed and synthesized at Xavier University involves training of Xavier researchers and students in drug target validation, biological assays of drug efficacy, evaluation of resistance pathways, and identification of synergistic drug combinations. At Xavier University, Dr. Thomas Wiese is an expert in computer-aided drug design methods, Dr. Maryam Foroozesh is an expert in the design and synthesis of small biologically active organic compounds, and Dr. Jayalakshmi Sridhar is an expert in both fields. On the Tulane Cancer Center side, Dr. Frank Jones is an expert in HER2 positive breast cancers and has an active drug validation program, and Drs. Barbara Beckman and Matthew Burow are experts in the areas of endocrine- and chemo-resistant breast cancers. The distinct contributions of each institution places this collaboration in a unique position to successfully design and validate drugs to address the most pressing challenges in breast cancer therapy. The specific aims of this collaborative project are to develop, promote, and sustain independent, competitive breast cancer research at Xavier University of Louisiana while developing a true partnership between the two institutions.

BODY:

Foroozesh/Beckman/Burow Subproject (Novel Ceramide Analogs as Anti-Cancer Agents)

The research accomplishments of this subproject include the following syntheses and bioassays:

Task 1- Hire research associate to assist in project. (Month 1)

Dr. Jiawang Liu has been working on the DoD project since the beginning of this grant project. He is an expert in the design, synthesis, and bioassays of biologically active molecules. We also had two Xavier Alumni as research technicians at different times in the past year.

Task 2- Identify student(s) to assist in project. (Months 1-3)

Over the past four years, a number of Xavier Undergraduate students (15) have been involved in this project.

Task 3- Design and synthesize new ceramide mimicking agents in order to perform structure-activity studies of these novel compounds in hopes of determining important and essential structural features leading to enhanced apoptosis induction. (Months 1-48)

Year 1: The synthesis of ceramide analogs (401, 402, 403, 404 and 406) with different backbones were achieved based on the previously published methods and reported in the Year 1 Progress Report.[1, 2] These specific analogs were designed to help us identify the contributions of the ceramide backbone in the anti-cancer activities. The synthetic scheme for this group of analogs is shown in Figure 1.

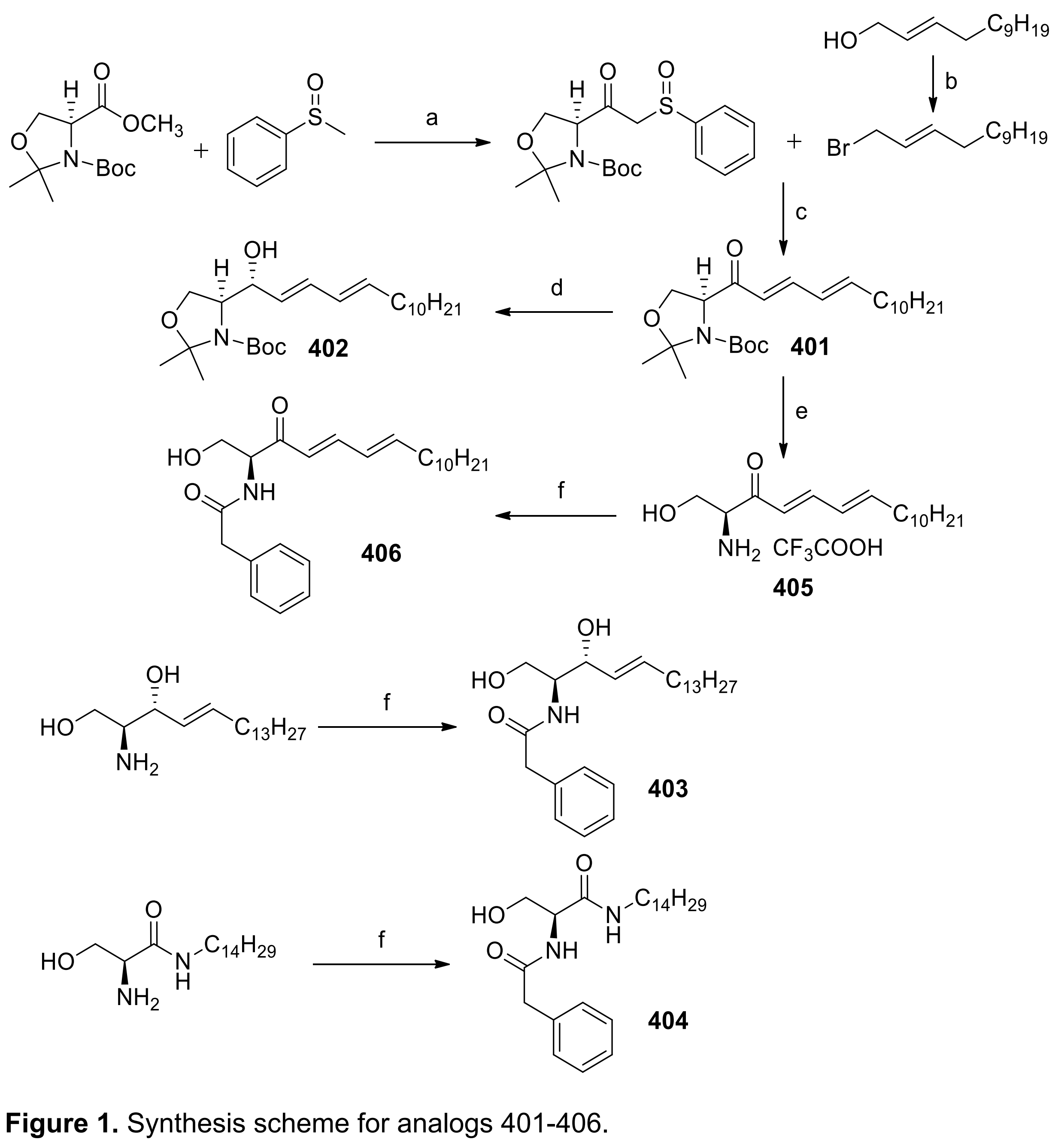


Figure 1. Synthesis scheme for analogs 401-406.

Ten ceramide analogs containing the imine group (409, 410, 412, 413, 415, 416, 417, 3T1, 3T2, and 3T3) were also prepared through a facile reaction.[3] Since one of our previously synthesized ceramide analogs containing an imine group has shown the

most potent anti-cancer activities,[1] the modifications here are expected to increase the anti-cancer activity and/or provide us with important structure-activity relationship information. The structures of these compounds are shown in Figure 2.

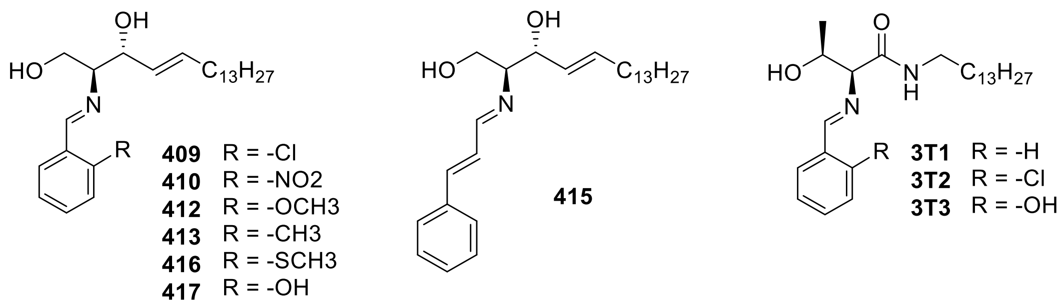


Figure 2. Structures of ceramide analogs 409, 410, 412, 413, 415, 416, 417, 3T1, 3T2, and 3T3.

A ceramide analog with the 1-hydoxy group blocked (503) was prepared using relatively inexpensive staring materials and simple synthetic route as shown in Figure 3. Compared with the previous synthetic methods,[2] our new synthetic route avoids the usage of expensive starting material methyl (S)-(-)-3-Boc-2,2-dimethyl-4-oxazolidinecarboxylate and excessive protecting and de-protecting steps. Using the starting material O-benzyl-serine, the 1-position blocked ceramide analog (503) was synthesized through a facile four-step route shown below.

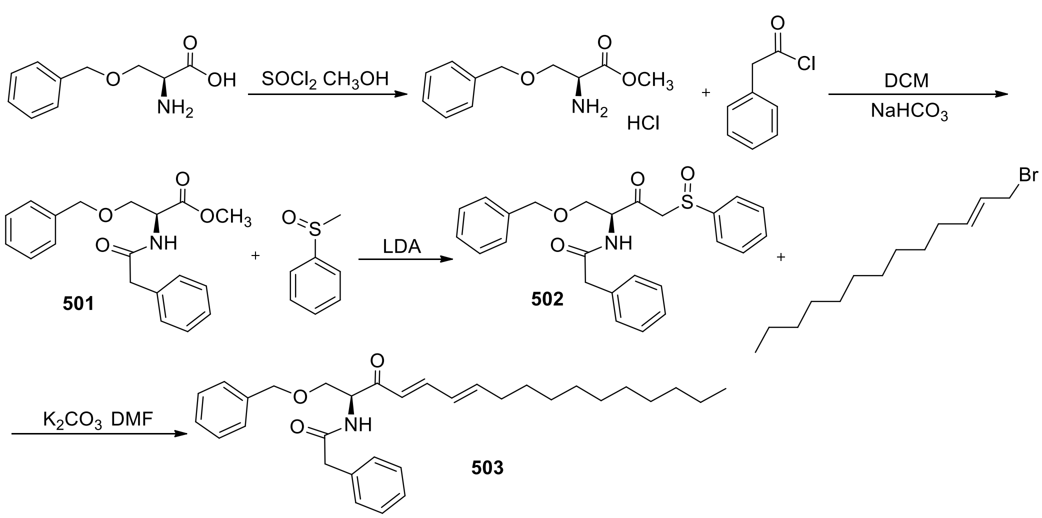


Figure 3. The four-step synthetic route used for the synthesis of analog 503.

Year 2: In Year 2, we focused on the synthesis of fluorescent building blocks for incorporation into the new ceramide analogs. Evidence shows that rigid modification of ceramide backbone enhances pro-apoptotic efficacy.[4,5] Employing flavone and coumarin cores as rigid moieties, the long conjugated system-modified ceramide

analogs were designed. The synthetic route used is presented in Figure 4. Incorporation of the long conjugated system adds molecular rigidity and fluorescence to the ceramide, facilitating the determination and tracking of the ceramide analogs in bioassays.

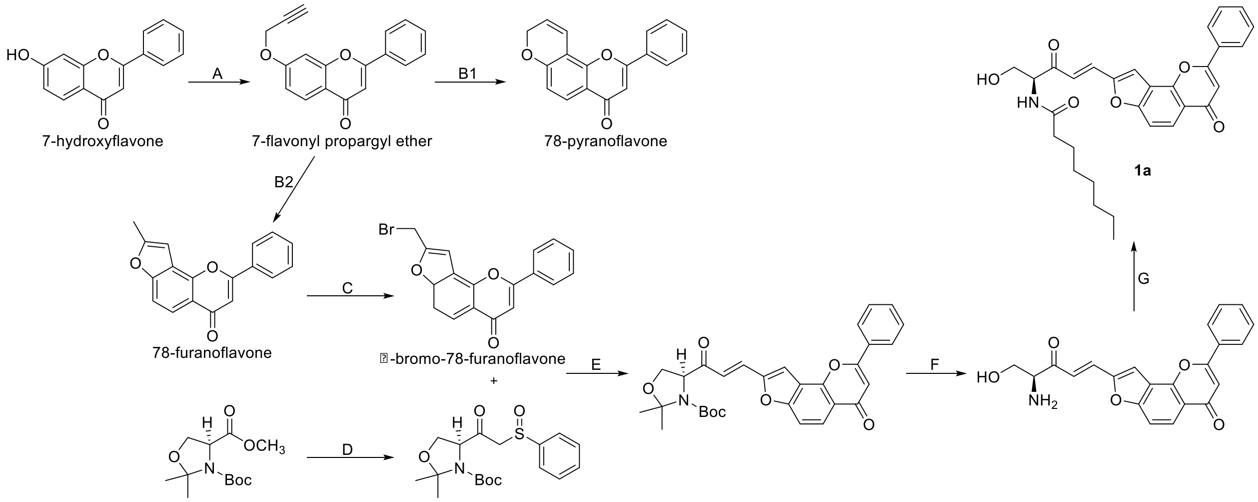


Figure 4. Synthetic route for flavonyl-modified ceramide analogs. Step A) propargyl bromide, potassium carbonate/acetone; Step B1) 200°C in N,N-diethylaniline; Step B2) 200°C cesium fluoride in N,N-diethylaniline; Step C) liquid bromine, aluminum chloride/dichloromethane; Step D) methyl phenyl sulfoxide, lithium diisopropylamine /tetrahydrofuran; Step E) potassium carbonate/N,N-dimethylformamide; Step F) trifluoroacetic acid/dichloromethane; and Step G) octanoic acid, DCC, HOBT/tetrahydrofuran.

In Year 2, we successfully synthesized 11 pyranoflavone and 4 furano flavone/coumarin building blocks for the preparation of fluorescent ceramide analogs (Figure 5). Through altering the reaction condition of Claisen rearrangement, pyrano- and furano-derivatives were obtained in a facile method and good yields.

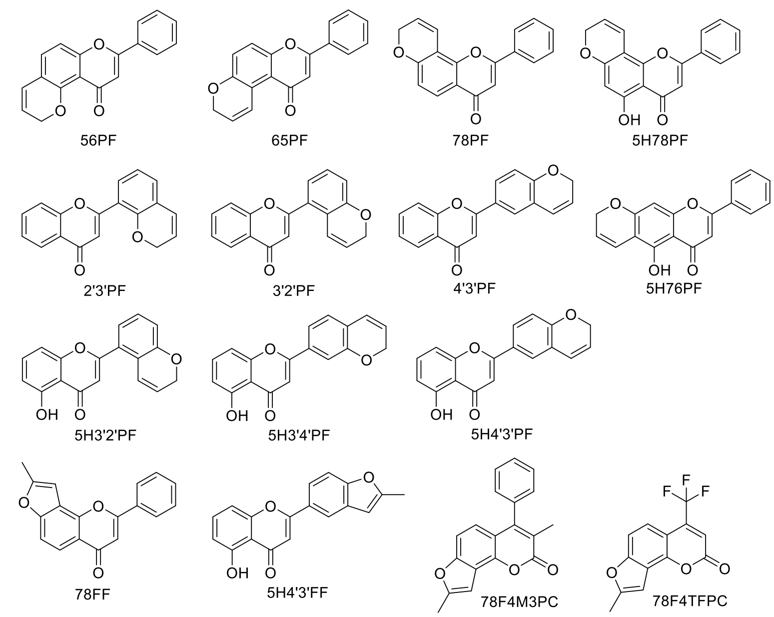


Figure 5. The molecular structures of pyranoflavones, furanoflavones, and furanocoumarins synthesized.

In Year 2, we also focused on the determination of molecular conformation of oxazolidine ceramide analogs. Oxazolidine ceramide analogs have shown high anticancer activity in previous studies. Mechanism investigations show that they could induce apoptosis as well as inhibit sphingosine kinase, a ceramide-metabolizing enzyme.[6] Because of the cyclization of 1-position OH group and 3-position amino group, the oxazolidine ceramide analogs possess considerable rigidity and fixed conformations, which are useful for investigating their interactions with molecular targets. Ceramide analogs, 401 and 402 are oxazolidine ceramide analogs. Thus, a conformational investigation of these compounds was carried out in Year 2. 1D NMR spectra show the two sets of signals for each compound. 2D NMR spectra clarify the stereochemistry of compounds. Through molecular simulation and conformational analysis, dual-conformation model of oxazolidine ceramide analogs is generated as seen in Figure 6.

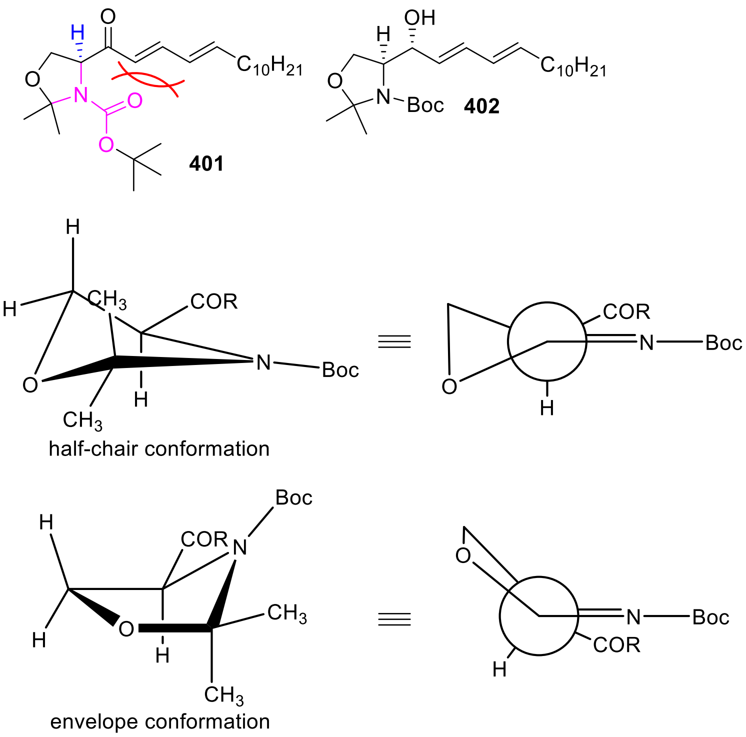


Figure 6. Hypothesized conformations for the conformational isomers of analogs 401. The bulky group $–COCH{=}CHCH{=}CHC_{10}H_{21}$ is located above the amide bond plane in the half-chair form, while below the amide plane in the envelope form. In both conformations α-H is in an axial position.

The sp^2 hybridization of N in the amide group results in a planar amide functional group (pink residue). Due to the presence of a bulky group ($–COCH{=}CHCH{=}CHC_{10}H_{21}$) on the carbon next to the N in the five-membered oxazoline ring, steric effects lead to the $–COCH{=}CHCH{=}CHC_{10}H_{21}$ group locating in the space above or below of the amide bond plane. Since these two poses cannot freely interchange, two relatively stable conformations are formed which are reflected by the two sets of signals in the NMR spectra. Figure 6 shows the two possible conformations of 401 in half-chair and envelope forms, respectively. In both conformations the α-H (in blue) is axial, which is consistent with the observations in ^1H NMR spectra. This new finding, disclosing the conformational isomerism phenomenon of oxazolidine.

Year 3: In Year 3, we successfully synthesized 11 pyrano-, furano-, dioxolo-, and pyridino-flavone/coumarin building blocks (Figure 7). We have established an effective method to synthesize flavone and coumarin derivatives, which could be used as fluorescent blocks of ceramide analogs.

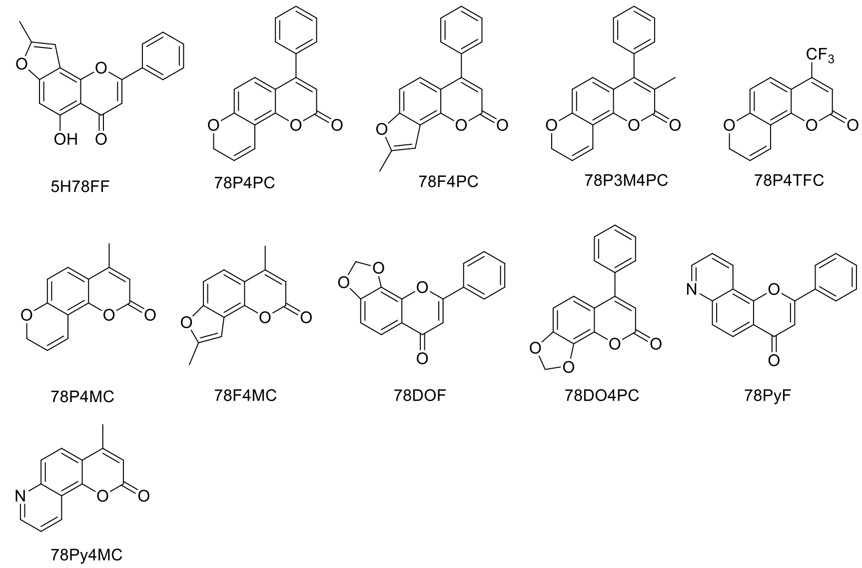


Figure 7. The molecular structures of 11 pyrano-, furano-, dioxolo-, and pyridino-flavones or coumarins synthesized.

In Year 3, we also successfully synthesized 6 ethynylflavones (Figure 8) as cytochrome P450 inhibitors. Enzymatic inhibition assays show that 4-ethynylflavone and 6-ethynylflavone are highly potent and selective inhibitors of P450 1A1 (K_i values of 0.035 and 0.039 μM). Since P450 1A1 is responsible for the conversion of environment pollutants to carcinogens in human body, 4-ethynylflavone and 6-ethynylflavone are potential cancer prevention agents.

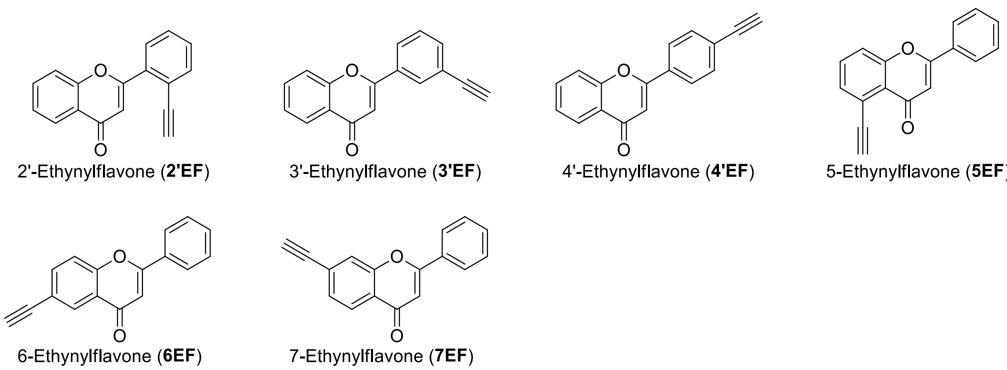


Figure 8. The molecular structures of ethynylflavones.

Task 4- Determination of Anti-Cancer Activities of the Ceramide Analogs. (Months 3-60)

The anti-cancer activities of ceramide analogs 401, 402, 403, 404, and 406 were tested using a cellular viability assay and a clonogenic survival assay in MCF-7, MDA-MB-231, and MCF-7TN-R cells. Compounds 401 and 406 were the most effective compounds across all cell lines with IC_{50} values of 4.05 ± 1.3 μM ($p<0.001$) and 4.26 ± 1.48 μM

(p<0.001) respectively, in the chemo-resistant MCF-7TN-R cell line. Interestingly, IC_{50} values for all analogs except analog 401 were lower in the chemo-resistant MCF-7TN-R and hormone therapy-resistant MDA-MB-231 cell lines, indicating that these compounds exhibit increased therapeutic potential in drug-resistant cancers (Table 1). The fact that two compounds with the 3-ketone-4,6-diene backbone (406 and 401) have shown the most potent anti-cancer activities in this group suggests that the 3-hydroxy-4-ene backbone is not necessary for bioactivity of ceramides as previously believed.[4] The raw results (Figures 9 and 10) are shown bellow.

Our results in apoptosis assays show that analog 406 induces a 4.3 ± 1.1 fold ($p<0.05$) increase over control in the induction of apoptosis, compared to C8-Cer with a 2.34 ± 0.79 fold increase. Analog 406-induced cell death is mediated through the intrinsic apoptotic pathways, with 3.59 ± 0.45 ($p<0.05$) fold increase in caspase-9 activity following treatment with the analog. In conjunction with our previous studies, these results suggest that development of ceramide analogs with a diene component in the sphingosine backbone may be well suited for the treatment of chemo-resistant breast cancer.

Table 1. IC_{50} values of ceramide analogs in the MTT viability assay and the clonogenic survival assay (μM). The values are the means of three independent experiments.

	IC_{50} values in viability assay (μM)			IC_{50} values in survival assay (μM)		
	MCF-7	MCF-7-NTR	MDA-MB-231	MCF-7	MCF-7-NTR	MDA-MB-231
401	3.906	4.047	26.76	5.07	1.854	1.454
402	26.44	9.908	45.54	5.692	5.185	3.174
403	37.28	4.742	35.79	4.175	5.62	1.585
404	233.6	28.96	NE	10.16	10.05	17.55
406	22.03	4.263	81.94	3.403	1.808	1.402

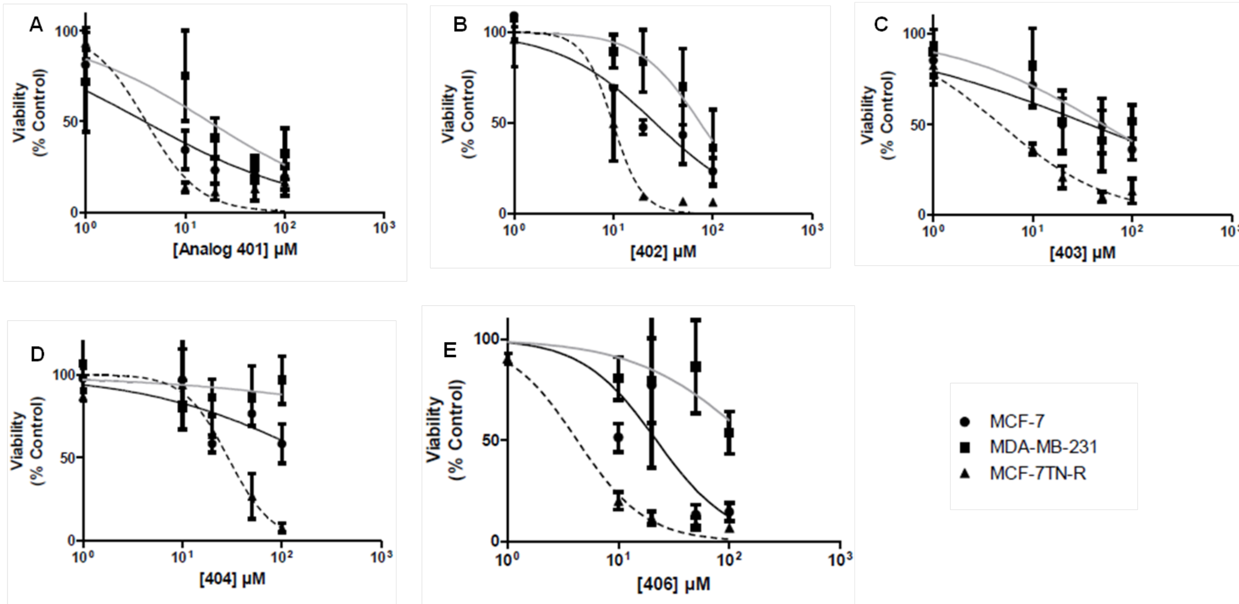


Figure 9. Effect of ceramide analogs on breast cancer viability. MCF-7, MDA-MB-231, and MCF-7TN-R cells were treated with increasing concentrations of analogs for 24h. The values are the mean ±SE of three independent experiments.

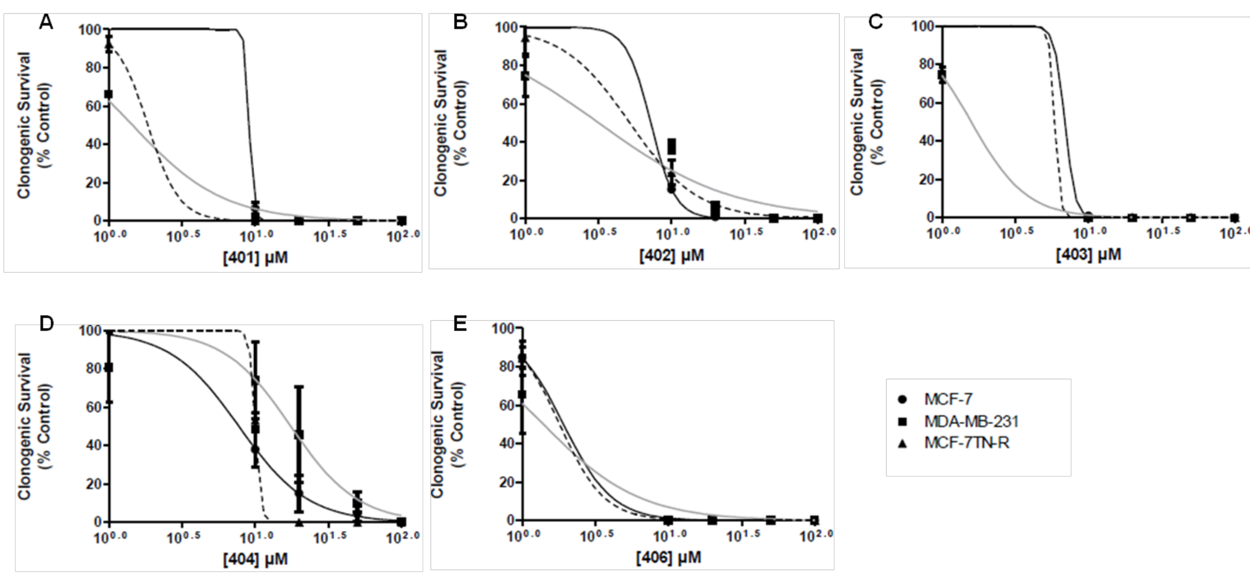


Figure 10. Effect of ceramide analogs on breast cancer clonogenic survival. MCF-7, MDA-MB-231, and MCF-7TN-R cells were treated with increasing concentrations of analogs and allowed to grow until colony formation was noted (generally 10-12 days). The values are the mean ± SE of three independent experiments.

A longitudinal activity comparison of analogs 315, 406, 415 and 503 was performed. These cell viability assays were performed on NCI/ADR-RES, NCI/ADR, OVCAR8, MCF-7, and MCF-7/Dox cells. The results showed that among these analogs, compounds 406 and 415 show the most potent activities. The raw data is provided bellow in Figure 11.

Glucosylceramide synthase (GCS) inhibition assays showed that analog 406 has a mild or no GCS inhibition activity in OVCAR8, NCI/ADR-RES, and NCI/ADR cells. This observation suggests that cytotoxicity of analog 406 is not a result of the inhibition of GCS enzyme. On the other hand, analog 503 showed a significant GCS inhibition activity in all of the tested cell lines. This observation confirms our hypothesis that GCS activity can be inhibited through modification of ceramide's 1-postion. These results provide us with a great perspective for designing novel inhibitors of GCS, an enzyme considered to be critical in cancer drug-resistance. The results of the GCS activity assays are shown bellow in Figure 12.

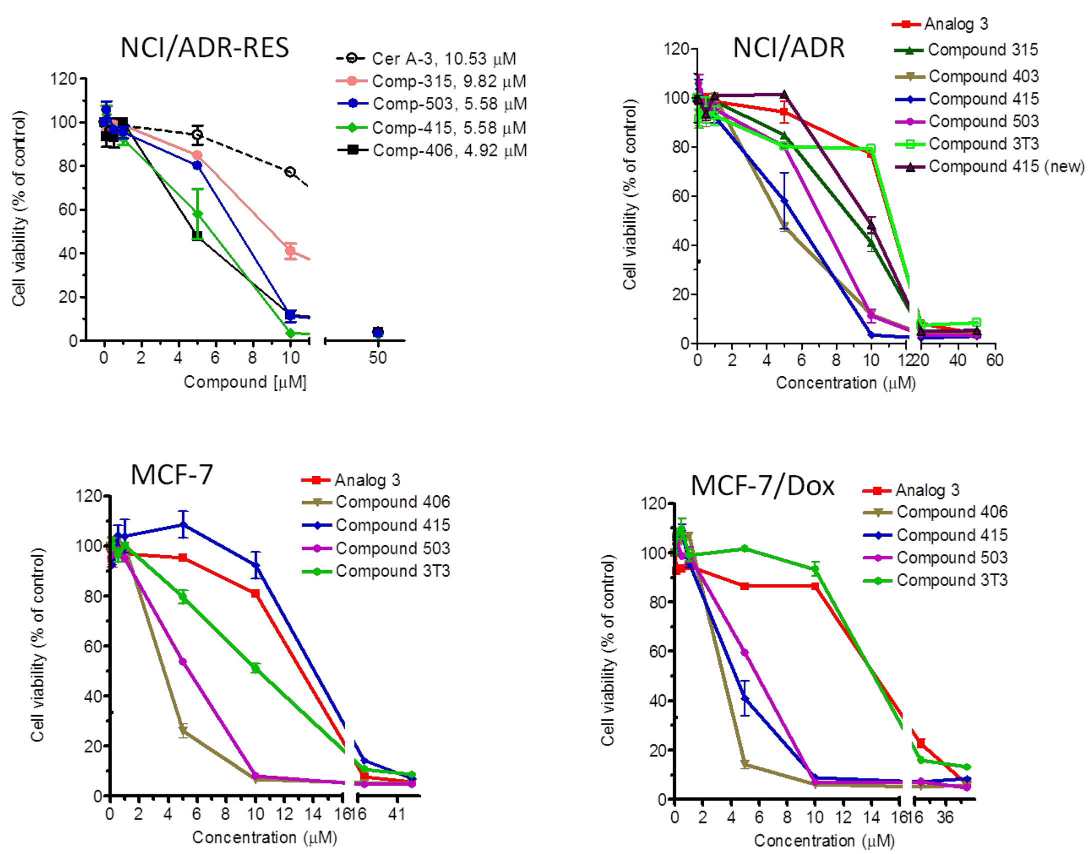


Figure 11. Effect of ceramide analogs on cancer viability. NCI/ADR-RES, NCI/ADR, MCF-7, and MCF-7/Dox cells were treated with increasing concentrations of analogs for 72 h. Analyzed by CellTiter-Glo luminescent cell viability assay from the Promega.

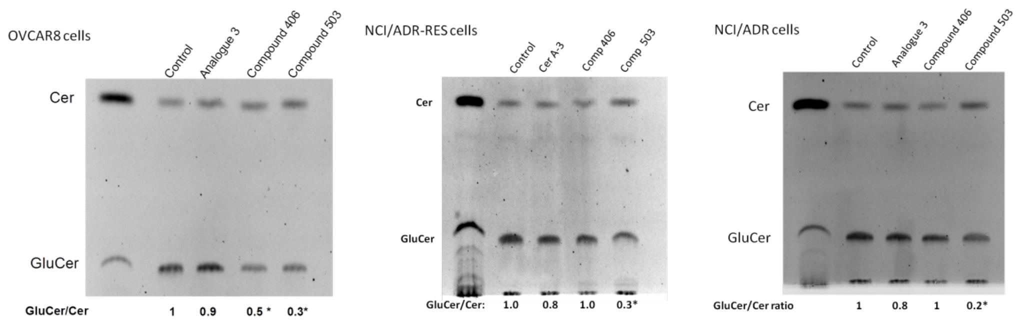


Figure 12. Results of GCS inhibition activity assays in OVCAR8, NCI/ADR-RES, and NCI/ADR cells demonstrated by thin layer chromatograph (TLC). Treatments for 48 hours in 5% FBS RPMI-1640 medium; analyzed by fluorescence enzymatic assay (Gupta V et al *J Lipid Res* 2010, 51:866-74), three times.

Year 4: In Year 4, we changed direction and focused on synthesizing and testing 32 flavone and coumarin derivatives for the inhibition of P450s 1A1, 1A2, and 1B1. The results are listed in Figure 13 & 14 and Table 2 & 3.

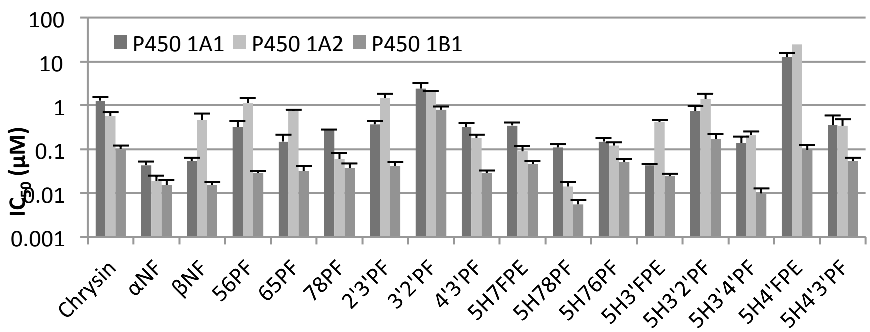


Figure 13. The IC_{50} values of pyranoflavones for inhibition of P450s 1A1, 1A2, and 1B1.

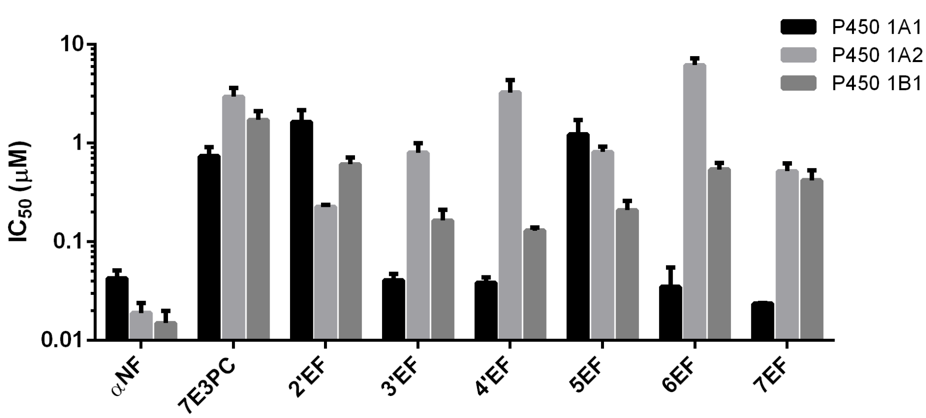


Figure 14. IC_{50} values of ethynylflavones for the inhibition of P450s 1A1, 1A2, and 1B1.

Table 2. Inhibitory activity and selectivity of flavone derivatives toward P450 1A2.

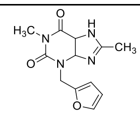
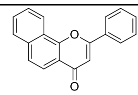
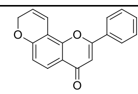
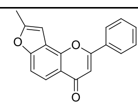
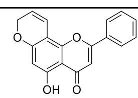
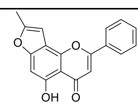
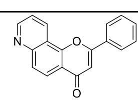
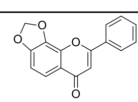
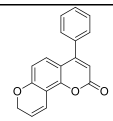
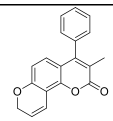
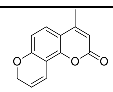
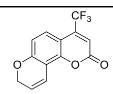
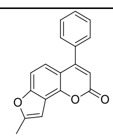
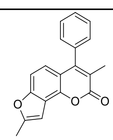
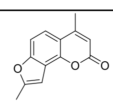
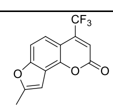
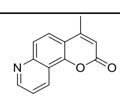
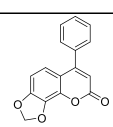
Compound	Short Name	Structure	K_i (μM)			Selective Index (SI)	
			1A1	1A2	1B1	1A1/1A2	1B1/1A2
Furafylline			>200	68.0 ±13.2	>200	>2.9	>2.9
α-Naphtho flavone	αNF		0.045 ±0.010	0.020 ±0.005	0.016 ±0.002	2.3	0.8
2	78PF		0.27 ±0.02	0.058 ±0.018	0.053 ±0.032	4.7	0.9
3	78FF		0.43 ±0.19	0.030 ±0.002	0.18 ±0.05	14	6.0
5	5H78PF		0.11 ±0.02	0.014 ±0.004	0.0056 ±0.0020	7.9	0.4
8	5H78FF		0.47 ±0.12	0.044 ±0.023	0.10 ±0.06	11	2.3
11	78PyF		0.040 ±0.008	0.076 ±0.011	0.12 ±0.02	0.5	1.8
12	78DOF		1.23 ±0.22	0.090 ±0.034	0.48 ±0.21	14	5.3

Table 3. Inhibitory activity and selectivity of coumarin derivatives toward P450 1A2.

Compound	Short Name	Structure	K_i (μM)			Selective Index (SI)	
			1A1	1A2	1B1	1A1/1A2	1B1/1A2
17	4P78PC		25.2±2.6	1.29±0.33	5.04±0.76	20	3.9
18	3M4P78PC		18.4±0.4	1.72±0.27	21.2±2.3	11	12
19	4M78PC		110±14	1.51±0.18	12.0±3.0	73	7.9
20	4TF78PC		60.6±6.2	0.39±0.08	20.4±4.8	155	52
21	4P78FC		12.0±0.3	5.02±0.16	4.62±0.78	2.4	0.9
22	3M4P78FC		11.6±0.4	3.70±0.73	22.6±6.1	3.1	6.1
23	4M78FC		37.9±5.3	1.15±0.17	7.46±0.52	33	6.5
24	4TF78FC		60.7±9.5	1.35±0.13	46.8±9.0	45	35
27	4M78PyC		101±17	14.2±4.0	46.6±9.1	7.1	3.3
28	4P78DOC		>200	3.55±0.84	30.1±4.6	56	8.5

Through the enzymatic studies, we successfully identified the selective P450 1A1 inhibitors 4'-ethynylflavone and 6-ethynylflavone, and the selective P450 1A2 inhibitors 7,8-furanoflavone and 7,8-pyrano-4-trifluoromethylcoumarin. 6-Ethynylflavone possesses a K_i value of 0.035 μM for P450 1A1, 177- and 15-folds lower than those for P450s 1A2 and 1B1, respectively. While, 8-pyrano-4-trifluoromethylcoumarin shows high selectivity for the inhibition of P450 1A2 with a K_i of 0.39 μM, 155- and 52-folds lower than its K_i values against P450s 1A1 and 1B1, respectively.

Furthermore, 7,8-pyrano-4-trifluoromethylcoumarin does not activate aryl hydrocarbon receptor when the concentration is lower than 1 μM, suggesting that this compound would not up-regulate AhR-caused P450 enzyme expression. *In cell* P450 1A2 inhibition assays show that 7,8-pyrano-4-trifluoromethylcoumarin decreases the MROD activity in HepG2 cells at concentrations higher than 1 μM. Thus, using 7,8-pyrano-4-trifluoromethylcoumarin, a selective and specific P450 1A2 action suppression could be achieved, indicating the potential for the development of P450 1A2-targeting cancer preventive agents.

Wiese/Burow Subproject (Identification of novel estrogens and antiestrogens in the USDA Phytochemical and FDA Marketed Drugs databases)

Research accomplishments of this subproject include the following tasks in Specific Aim 1: ***Develop structure-based pharmacophore models and ligand-receptor (docking) models for estrogens based on the crystal structures of ER alpha and beta (with bound agonists or antagonists) and then virtually screen the USDA Phytochemical, Chinese Herbal Medicine, and the FDA Marketed Drug Databases for new estrogens.***

Task 1- Identify student to assist in project. (Month 1)

Year 1: Pharmacy students Chioma Obih and Felicia Gibson who have worked in the Wiese lab for the previous 2 years were assigned to this project in Fall 2011. Both students were supported by the College of Pharmacy Center of Excellence Grant. Dr. Wiese trained Ms. Obih on structure-based modeling methods using the MOE software and she worked with Dr. Wiese on Task 2. Ms. Gibson focused on working with Ms. Candace Hopgood on *in vitro* bioassays.

Year 2: Ms. Obih graduated in Spring 2013 and left the group. Starting January 2013, Ms. Gabriela Barbarini, a pharmacy exchange student from Brazil started working in the Wiese lab learning bioassay techniques used in Task 6. Ms. Barbarini was a third year pharmacy student doing one year in the US at Xavier's college of pharmacy and selected to work in the Wiese lab. During summer of 2013, Ms. Barbarini worked in the

laboratory of Dr. Burow at Tulane (DOD Project Collaborator of Dr. Wiese) and learned additional bioassay methods. In Fall 2013, she was back in the Wiese lab, applying her skills to evaluate the estrogen activity of compounds identified in this project in a 3 credit research experience course.

Year 3: Ms. Barbarini completed her fall semester research experience where she performed reporter gene assays on the 8 stilbenes identified in the initial screen as having some estrogen activity. This produced dose response data indicating the relative potency of these compounds. She then finished her exchange program and returned to Brazil in December 2013. A new P1 pharmacy student, Ms. Tamara Mitchell, joined the lab in Fall 2013 and quickly learned cell culture techniques and then applied these skills to testing the 8 stilbenes shown to have estrogen activity in the MCF-7 breast cancer cell proliferation assay. This produced dose response data for these compounds. Tamara then presented this data in a poster at the Spring 2014 ACS meeting. In spring 2014, Tamara started to learn molecular modeling methods relevant to the Project and will apply these skills to modeling analysis of the stilbene data in Y4.

Year 4: Ms. Tamara Mitchell, Pharmacy student, continued to work on this project in Y4 focusing on modeling of the stilbenes identified as active in the estrogen receptor ligand binding site. In addition, Mr. Gerald Guirard, pharmacy student, started working on the Project in December 2014. Both students focused on modeling aspects with Tamara refining ligand-receptor interaction models for the stilbenes and Gerald developing pharmacophore models to screen the National Pharmaceutical Collection database. Tamara and Gerald worked full time on the Project in Summer 2015 and both continue on a part time basis in fall 2015. To facilitate progress, both Tamara and Gerald were sent to Montreal, Canada in spring 2015 to obtain advanced training in molecular modeling methods at the annual Chemical Computing Group North American Users meeting.

Task 2- Develop structure-based pharmacophore models for estrogens. (Months 1-4)

2a - Obtain all crystal structures of ER LBDs (Month 1)

Year 1: A search of the Protein Database in Fall 2011 resulted in the identification of 62 crystal structures of the human Estrogen Receptor (ER) ligand-binding domain (LBD), all of which contained one bound ligand. These LBD structures were processed and aligned relative to each other so that similarities and differences in ligand-binding pockets could be identified.

2b - Sort LBD structures by cavity shape and helix-12 position (Months 1-3)

In preliminary studies prior to this project, we have shown that ligand receptor docking (or virtual screening using docking) can produce very different results between ER LBD

structures containing steroid or stilbene ligands, even though both ligands are agonists and the LBD cavity sizes are very similar. The Xavier Molecular Structure and Modeling Core was utilized to compare the ligand-binding cavity sizes of the 62 processed structures. At the same time, a manual sorting was undertaken to group ER LBD crystal structures by bound ligand type, cavity size, and position of helix 12. This process resulted in the identification of 26 structures in the antagonist configuration (helix 12 open) and 36 structures in the agonist configuration with helix 12 closed. While cavity volume did not clearly group these structures, a combination of cavity size and bound ligand type was used to select representative agonist and antagonist crystal structures of the ER LBD. These 5 agonist structures (1ERE, 2G50, 2P15, 2QH6, and 3ERD) and 3 antagonist structures (1ERR, 3DT3, and 3ERT) will be used in the structure-based database screening.

Year 2: A computational mythology was identified that can utilize all of the ER LBD structures for the virtual screening rather than a subset of 5. The new strategy was to use as many of the 62 ER LBD structures as needed for docking the target databases based on the similarity of each database compound to the bound ligands of each available ER LBD. The critical part of this method involves sorting the databases by similarity to the bound ligands of all 62 ER LBD structures. Then, database members most similar to any bound ligand are docked into the corresponding ER LBD crystal structure. This approach should reduce false positives and negatives in the virtual screening since the ER LBD is known to take on slightly different ligand binding cavity shapes as it binds to different ligands. In order to apply this methodology to this project, Dr. Wiese attended a one-day training workshop held by Chemical Computing Group (Makers of MOE software) in May 2013. From this training, a process was developed to presort the databases before docking and the skills were obtained to carry out the process. This new workflow for the screening and validation process has been refined to the following:

1. Wash and Filter Databases to molecules that may bind to ER LBD (same as original plan).

2. Sort databases into subsets by similarity to the ligands bound to the 62 ER LBD structures.

3. Dock sorted database subsets into corresponding ER LBD that have been aligned by binding cavity.

4. Repeat steps 2 and 3 after Meteor-derived Phase I metabolites are generated.

5. Select compounds for validation with bioassays based on docking scores.

Year 3: This process is continued in Y3.

Year 4: In late 2014, a number of new crystal structures of the estrogen receptor ligand binding domain (ERLBD) were deposited in the Protein Database. Most of these included novel ligands with chemical structures quite different from the typical steroid estrogens. With these additions, the available ERLBD structures available to use in this project went from 62 to 86, with 76 of these containing nonsteroid ligands. These complexes were added to our database of ERKLBDs and it was determined that these new structures increased the ligand shape diversity significantly from the original 62 structures. Included in the new structures were ER complexes with resveratrol and other stilbenes as well as many other novel nonsteroids. These new structures significantly increased the diversity of ERLBD cavity shape and orientation of cavity amino acids from structures available previously. Thus, Tamara could now model the resveratrol analogues evaluated in the Project in the resveratrol-ER complex structure (as well as others). At the same time, the many novel ligands in these new ERLBD structures increased the potential for using ligand pharmacophore based database screening to find very novel estrogen acting compounds in the pharmaceutical database. Gerald then developed pharmacophore models for 14 of ERLBDs with the most diverse ligands and started using these to screen the National Pharmaceutical Database.

2c - Develop pharmacophore models from representative LBD structures (Months 1-4)

Year 2: Since all of the ligands in the selected ER LBD structures bind to the ER using similar interactions, the development of classical pharmacophore models for the ER LBD models was determined to be unnecessary. Structure-based screening for this project will utilize docking to the selected crystal structures where typical pharmacophore interactions are part of the ligand pose generation and score process.

Year 4: We used the expanded diversity of the ERLBDS available to us in Y4 to develop diverse pharmacophore models of ER ligands in the ER LBD.

The National Pharmaceutical Collection (NPC) database (version 2012) was obtained from the Therapeutically Relevant Informatics for Prioritization, Optimization, and Development (TRIPOD) informatics group within the Division of Preclinical Innovation, National Center for Advancing Translational Sciences, NIH. The NPC is a 2D structure database of 14,814 compounds with a variety of fields describing the CAS ID, drug name, reference and other information. The first step was processing the database to compounds that might reasonably fit into the ER. A conservative molecular weight filter of less than 2,000 was used to remove very large and all peptide drugs in the database. Molecules without any rings were then removed leaving 7,230 entries. These compounds were then washed (charge neutralized, counter ions and solvents removed). Finally, reasonable 3D structures were generated using CORINA followed by geometry optimization using the MMFF94 force field in MOE.

Pharmacophore models of 14 ERLBD complexes were made in MOE representing some of the most diverse ligands known to bind ER. These models use structure features of the bound ligand as well as the interaction sites in the corresponding ERLBD complex. The reduced NPC database was screened with each pharmacophore model producing a list of hits (Range: 2 to several hundred). These hits were then refined using ligand-receptor docking in the corresponding ERLBD structure producing the final screening hit list (see Task 4 below).

Task 3- Mine phytochemical and marketed drug databases with pharmacophore models. (Months 3-5)

3a - Evaluate Docking methods for virtual screening of estrogens (Months 4-8)

Year 1: The Xavier Molecular Structure and Modeling Core was utilized to quickly evaluate the potential for the MOE, Gold, Glide, and Surflex Dock to be used for docking into the ER LBD. FlexX is no longer used in this lab, and the Glide method is an additional method used by the Molecular Structure and Modeling Core. This fast study using default setting of the software to replace the bound ligand into the binding cavity did not identify significant differences in the performance of these methods. Considering that the Wiese lab has the most experience with the MOE software and the fact that the MOE software is the only docking package available to us that can include a force field optimization, MOE was selected for further optimization studies.

Year 2: Each ER LBD structure was evaluated and processed for docking using the MOE Structure Preparation Module.

3b - Ligand replacement optimization docking of representative LBD structures using MOE, Gold, FlexX, Surflex Dock (Months 4-7)

Year 1: A systematic ligand replacement study was performed using the MOE software, and an optimal configuration of the MOE docking method was identified that produced ligand replacement very close to the crystal structure (low RMSD). The MOE docking software, with its multiple ligand placement and scoring methods, was systematically evaluated for ability to replace the bound ligand in representative examples of the ER agonist and antagonist LBD structures.

Year 2: A method using the Triangle Matches Placement Method and the London dG scoring followed by a ligand minimization and final rescoring with method GBVI/WSA dG was found to produce ligand replacements very close to the crystal structure.

Task 4- Refine pharmacophore selection of estrogens using docking. (Months 6-8)

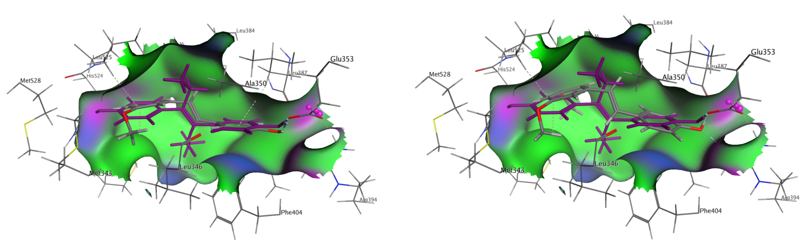
Year 1: Not initiated.

Year 2: The phytochemical (76,451 compounds) and marketed drug databases (16,096 compounds) were obtained from the Xavier Molecular Structure and Modeling Core in SDF format.

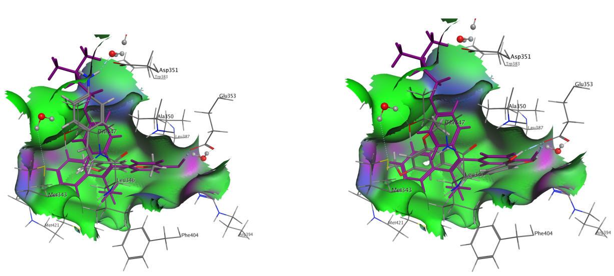
Year 3: The processing these databases for virtual screening continued in Y3. This includes creating all tautomers, isomers, enantiomers, and filtering out compounds too large to bind ER using MOE. In addition, the software Meteor will be used to create potential Phase I metabolites of each structure. The process of sorting these databases by similarity to the bound ligands of the 62 ER LBDs continues (see Task 2b above) and a new set of test compounds should be identified in first half of Y4.

The ER docking results for the 8 stilbenes found to have ER agonist or antagonist activity are shown below. Note that the stilbenes with agonist activity share the binding mode of the potent agonist diethylstilbestrol (DES) shown in dark magenta while the stilbenes with antagonist activity have only a partial overlap with the binding mode of 4OH-Tamoxifen (dark magenta). This binding mode allows the helix 12 of ER to close and initiate transcription. The antagonist stilbenes 4 and 9 are cis (bent) and occupy both the estradiol binding pocket and the channel created by the aryl amine of tamoxifen in the docking simulation. This is the binding mode of typical ER antagonists that directly push away the helix 12 of the ER by creating a new channel. Stilbene 28 is trans (not bent) and docking places the ligand in the estradiol binding pocket where it may act as a indirect ER antagonist and indirectly acting on helix 12 of ER.

ER Docking of Agonist Stilbenes 10 and 11 in 3ERD



ER Docking of Antagonist Stilbenes 4 and 28 in 3ERT



Year 4: Using the diverse ligands obtained from ERLBD complexes, 14 pharmacophore models were generated, each from a different ERLBD complex. The 14 pharmacophore models were then used to screen the NPC database obtaining hits. Hits from each pharmacophore based screen were then refined using ligand-receptor docking using methods developed in Task 3, Y2. Docking results were evaluated by interaction score and visual interaction in the ERLBD to obtain a final hit list of drug like molecules identified in the NPC that might have estrogen activity.

The pharmacophore guided, docking refined search of the NPC did identify all known estrogen agonists and antagonists in the database (Estradiol, Tamoxifen, etc.). Thus, the method does identify estrogens and antiestrogens. In addition to the known estrogens and antiestrogens, the method identified a number of other pharmaceutical compounds as potential estrogens. Of these, those that are in current use were selected for *in vitro* testing of activity in the carry forward year 5. These compounds are:

1. Sulfacetamide sodium (127-56-0): antibiotic ophthalmic solution
2. Propylparaben (94-13-3): preservative typically found in many water-based cosmetics, such as creams, lotions, shampoos and bath products
3. Dapsone (80-08-0): Antibiotic used in the treatment of leprosy, pneumocystic jiroveci, etc.
4. Polyethylene terephthalate (25038-59-9): most common thermoplastic polymer resin of polyester used in fibers for clothing, containers for liquids and foods
5. Benzocaine (94-09-7): Local anesthetic; antihermorrhoidal agent
6. Hordenine (539-15-1): alkaloid of phenethylamine class that occurs naturally in a variety of plants, not used, widely sold as an ingredient in nutritional supplements
7. Adrenalone (99-45-6): adrenergic agonist used as a topical vasoconstrictor, drug is largely obsolete
8. Ethylparaben (120-47-8): Antifungal preservative used in cosmetic database
9. Chlorphenesin (104-29-0): centrally acting muscle relaxant used to treat muscle pain and spasms. No longer used for this purpose in most developed nations
10. Acetaminophen (103-90-2): Analgesic; anti-pyretic
11. Methylparaben (99-76-3): Antifungal agent in a variety of cosmetics and personal-care products
12. Oxymetazoline (1491-59-4): Vasoconstrictor; imidazoline derivative; used in the treatment of nasal congestion and used in relief of eye redness
13. Lindane (58-89-9): Antiparasitic agent used in the treatment of head lice and scabies
14. Levomethadone (76-99-3): Synthetic opioid analgesic and antitussive which is marketed in Europe
15. Zelandopam (138086-00-7): A dopamine D1-like receptor agonist; still in clinical trials

16. Levorphanol (77-07-6): Analgesic; opioid; used in the treatment of moderate to severe chronic pain
17. Edrophonium (116-38-1): Acetylcholinesterase inhibitor used in the treatment of myasthenia gravis or reversal of nondepolarizing neuromuscular blocking agents

Task 5- Hire research associate to assist with *in vitro* assays. (Month 7)

Year 1: Ms. Candace Hopgood had worked in the Wiese lab for 2 years and was transferred to this project briefly in spring and summer of 2012. She spent the summer testing some of the bioassays to be used in the validation phase of this project including the Lantha Screen ER binding assay and the labs MVLN and T47D reporter gene assays. Ms. Hopgood left the Wiese lab in Fall 2012 to focus on her application to Xavier Pharmacy School where she is now a student.

Year 2: In January 2013, Ms. Peng Ma was partially reassigned to the bioassay component of the Project (50% effort). Ms. Ma also continues to work with Dr. Wiese in the RCMI (NIH-funded Research Centers in Minority Institutions) Cell and Molecular Biology Core (at 50% effort) that he directs. Ms. Ma is very skilled with most of the *in vitro* methods used to validate the virtual screening in this project. Ms. Ma has also been involved in training and working with Ms. Barbarini (a new student) in the lab.

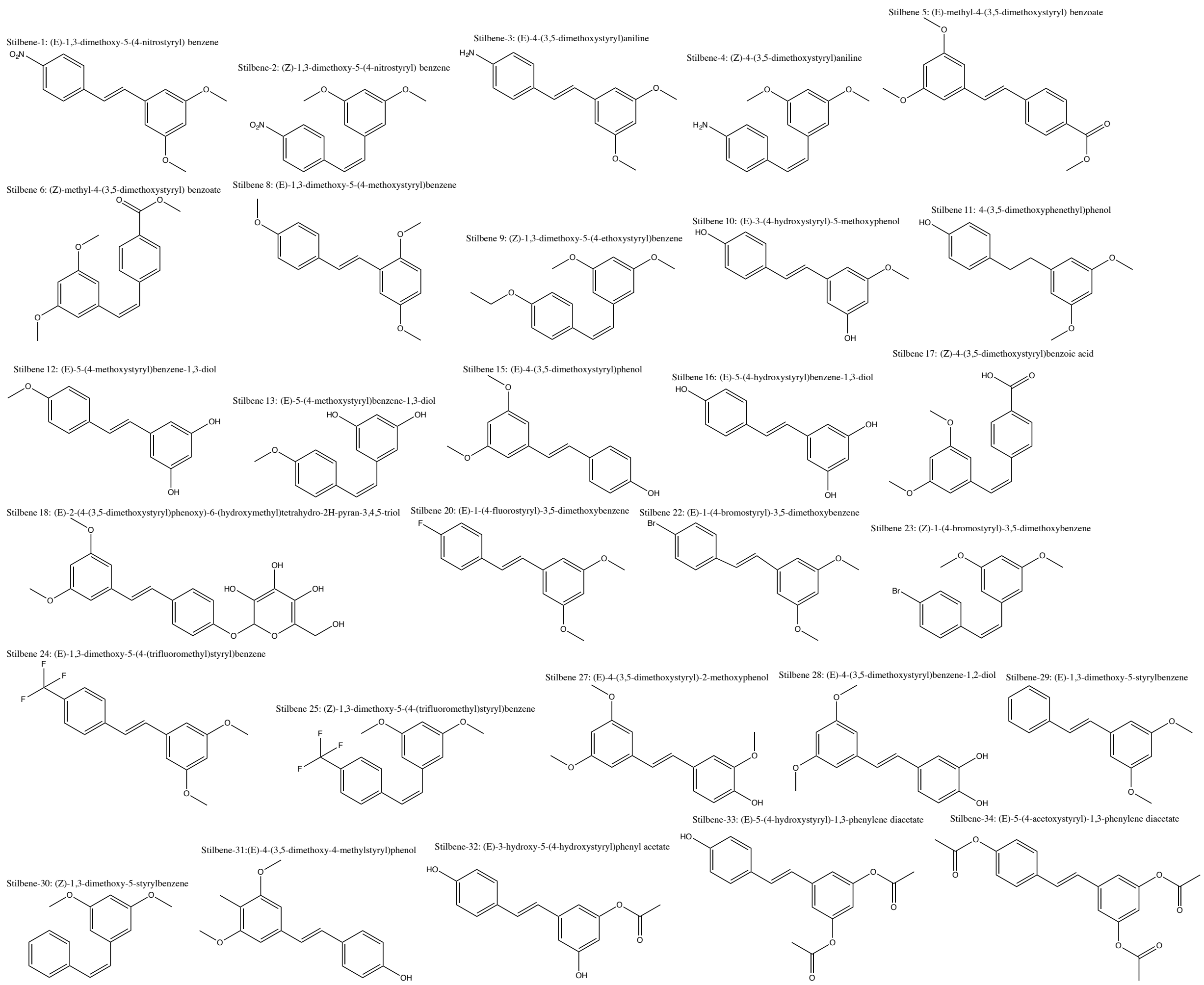
Year 3: Ms Ma continues to dedicate 50% of her time to this project maintaining cells, performing bioassays, training and working with students doing bio assays and learning the ER binding assay method.

Task 6- *In vitro* validation of estrogen activity. (Months 8-30)

6a - Obtain samples of 10-20 test compounds selected in virtual screen (Months 8-9)

Year 1: Not initiated.

Year 2: A preliminary database of 29 stilbene analogs has been obtained from the USDA Natural Products Utilization Research Unit in Mississippi. The stilbene structure core has been used as the basis for potent ER agonists and antagonists that are in the registered pharmaceuticals and herbal medicine databases. The 29 analogs obtained have been characterized for anticancer effects, but not evaluated for estrogen activity.



If these Stilbenes, 19 have a trans (extended) configuration like the well studied and potent estrogen agonist diethylstilbestrol (DES), one is a stilbane with no potential for E, Z isomers and nine are in the cis configuration where the overall structure is bent. We expect that some of the extended stilbenes will have estrogen agonist activity and that some of the bent (cis) stilbenes might have some antiestrogen activity.

6b - Perform FP ER-alpha/beta binding determinations of test compounds (Months 9-15)

Year 1: Not initiated.

Year 2: Dr. Wiese has been training Ms. Ma to carry out the ER binding assays and she has developed her proficiency to a level that she will soon run ER alpha and beta binding curves for the 29 stilbenes.

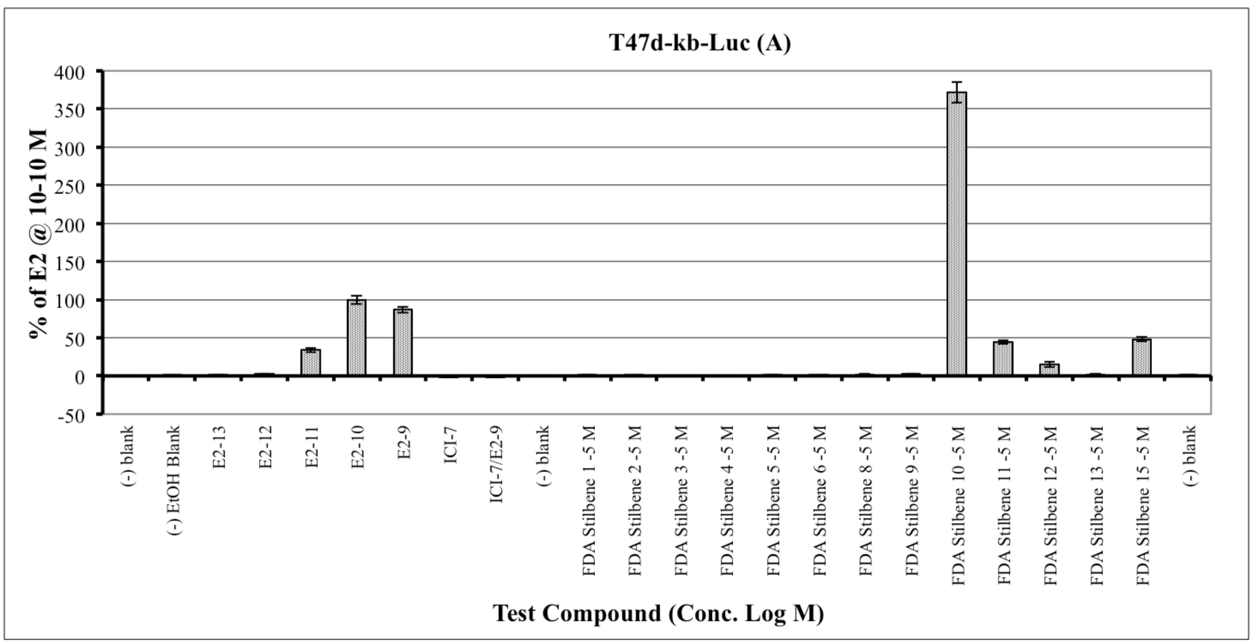
Year 3: ER binding was not completed for the 8 active stilbenes in Y3 due to problems with the FP instrument and data collection from the instrument in the Wiese Lab. A new computer workstation was obtained and binding determinations will be done n Y4. In

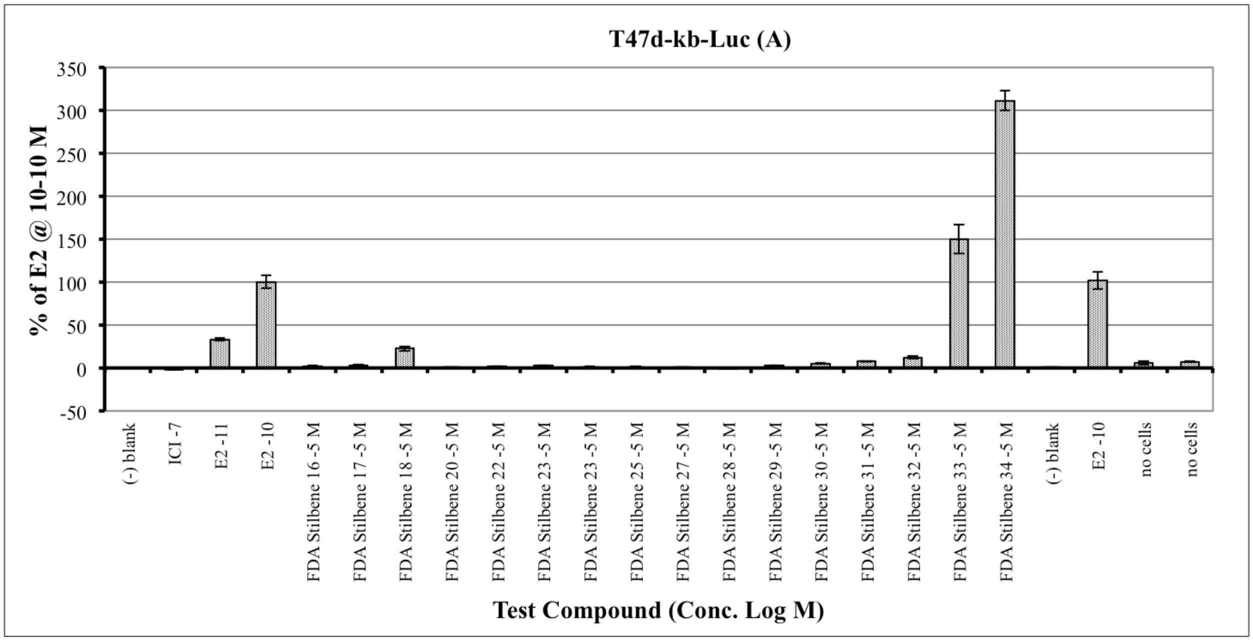
addition, a method development process has started where the FP binding assays can be read using the plate reader in the Xavier RCMI CMB core lab.

6c - Perform MVLN reporter gene agonist/antagonist determinations of test compounds (Months 12-18)

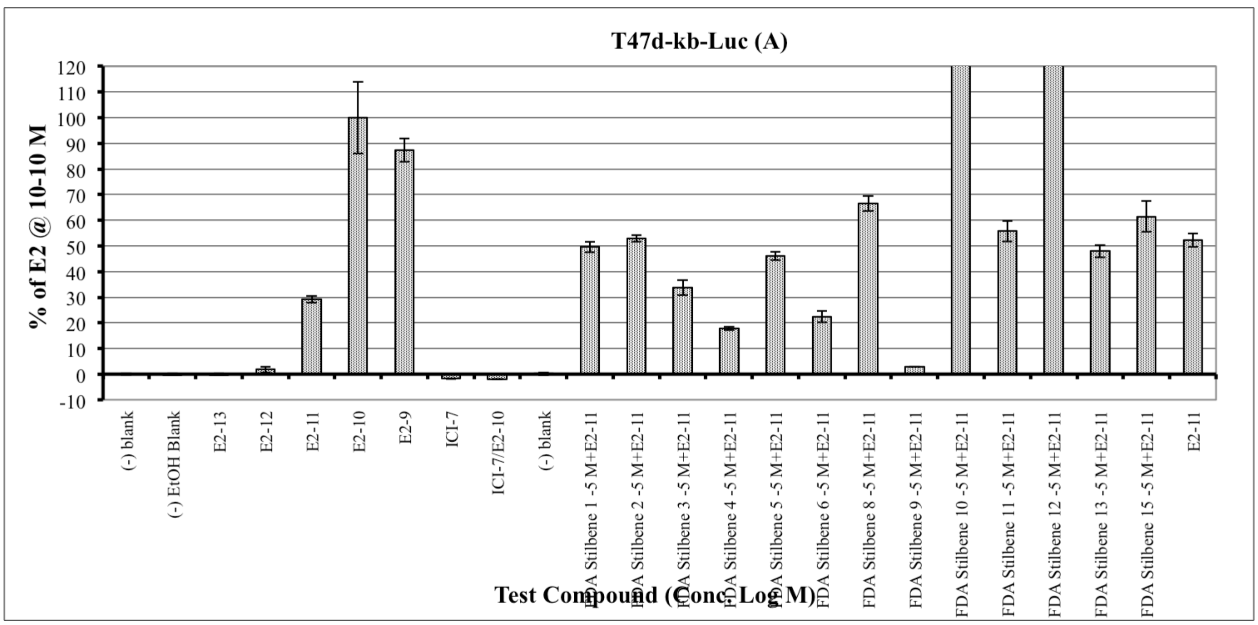
Year 1: Not initiated.

Year 2: The 29 stilbene compounds have been used as a test set to train and standardize the ER responsive reporter gene assays in the Wiese Lab. All compounds were tested in thee experiments at 10 uM in the T47Dkb-Luc cells to check for agonist and/or antagonist activity. Rather than using the MVLN reporter gene cells, the T47Dkb-Luc cells have been used since they provide a stronger estrogen response and since they are 10X more sensitive to estrogens. Representative data are shown below. Five stilbenes were found to be ER agonists, 3 exhibit antagonist activity and 7 stilbenes potentiate the activity of estradiol.

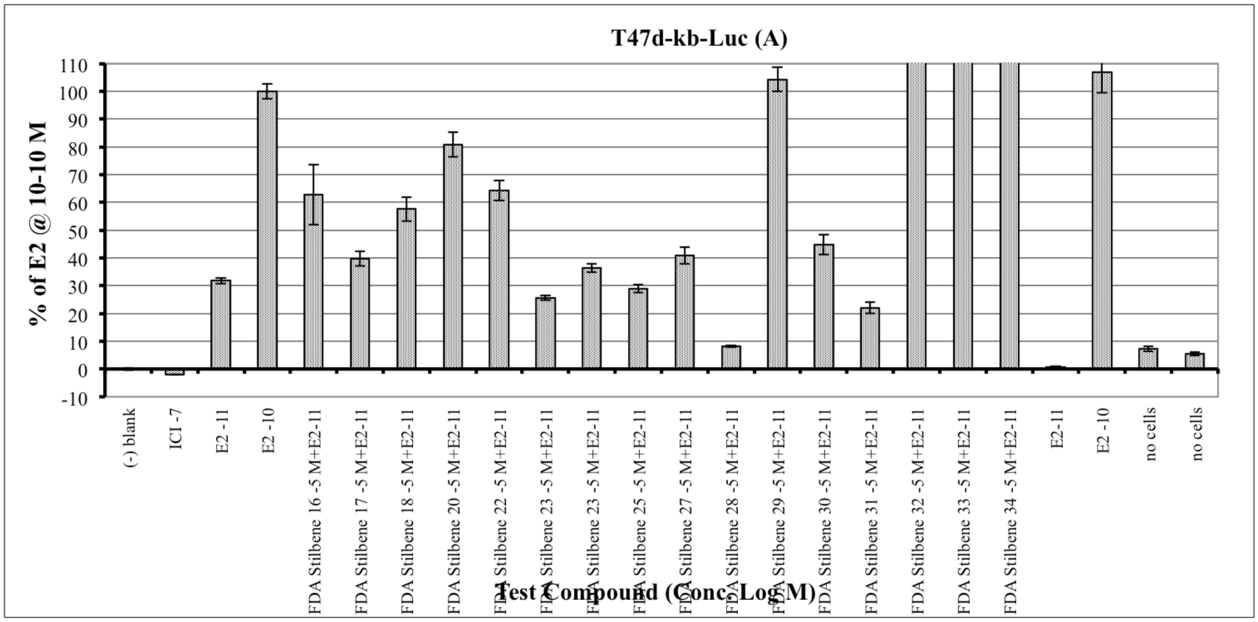




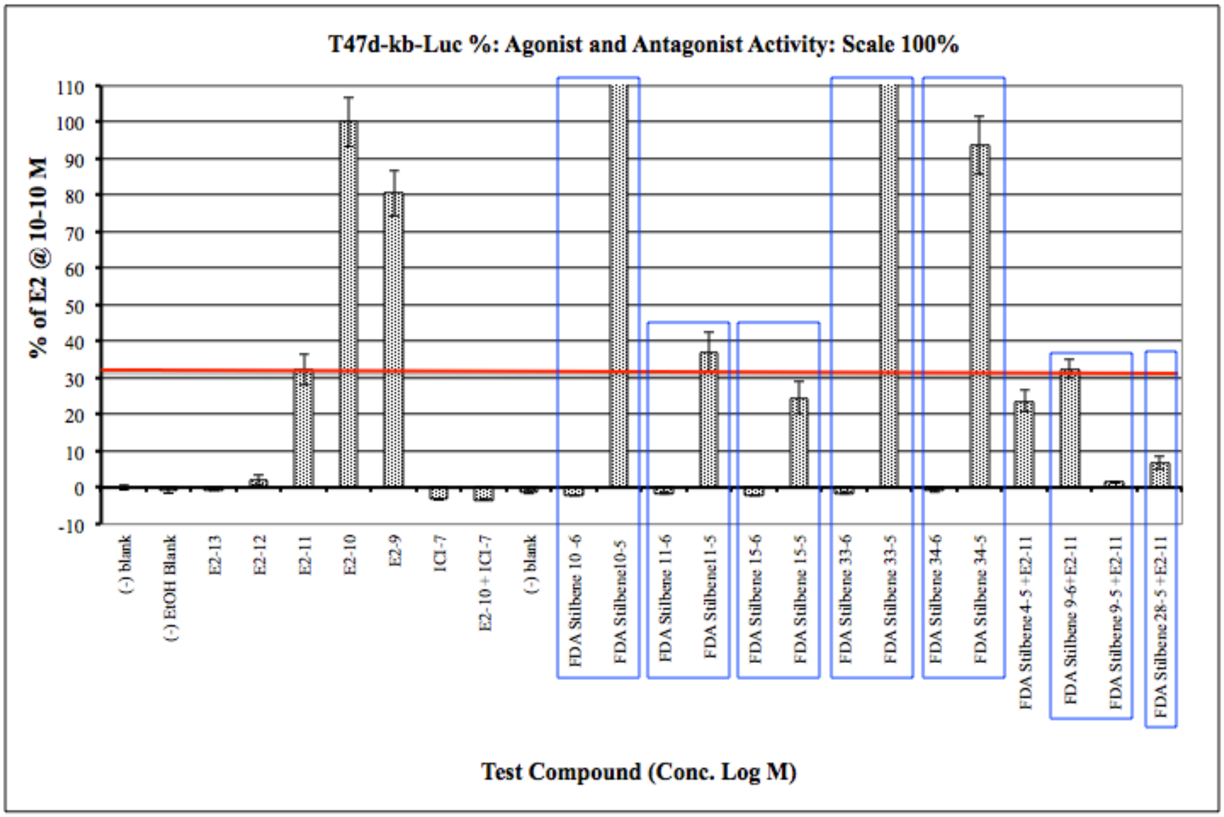
T47d-kb-Luc (A)
% of E2 @ 10-10 M
Test Compound (Conc. Log M)
350
300
250
200
150
100
50
0
-50
(-) blank
ICI -7
E2 -11
E2 -10
FDA Stilbene 16 -5 M
FDA Stilbene 17 -5 M
FDA Stilbene 18 -5 M
FDA Stilbene 20 -5 M
FDA Stilbene 22 -5 M
FDA Stilbene 23 -5 M
FDA Stilbene 23 -5 M
FDA Stilbene 25 -5 M
FDA Stilbene 27 -5 M
FDA Stilbene 28 -5 M
FDA Stilbene 29 -5 M
FDA Stilbene 30 -5 M
FDA Stilbene 31 -5 M
FDA Stilbene 32 -5 M
FDA Stilbene 33 -5 M
FDA Stilbene 34 -5 M
(-) blank
E2 -10
no cells
no cells

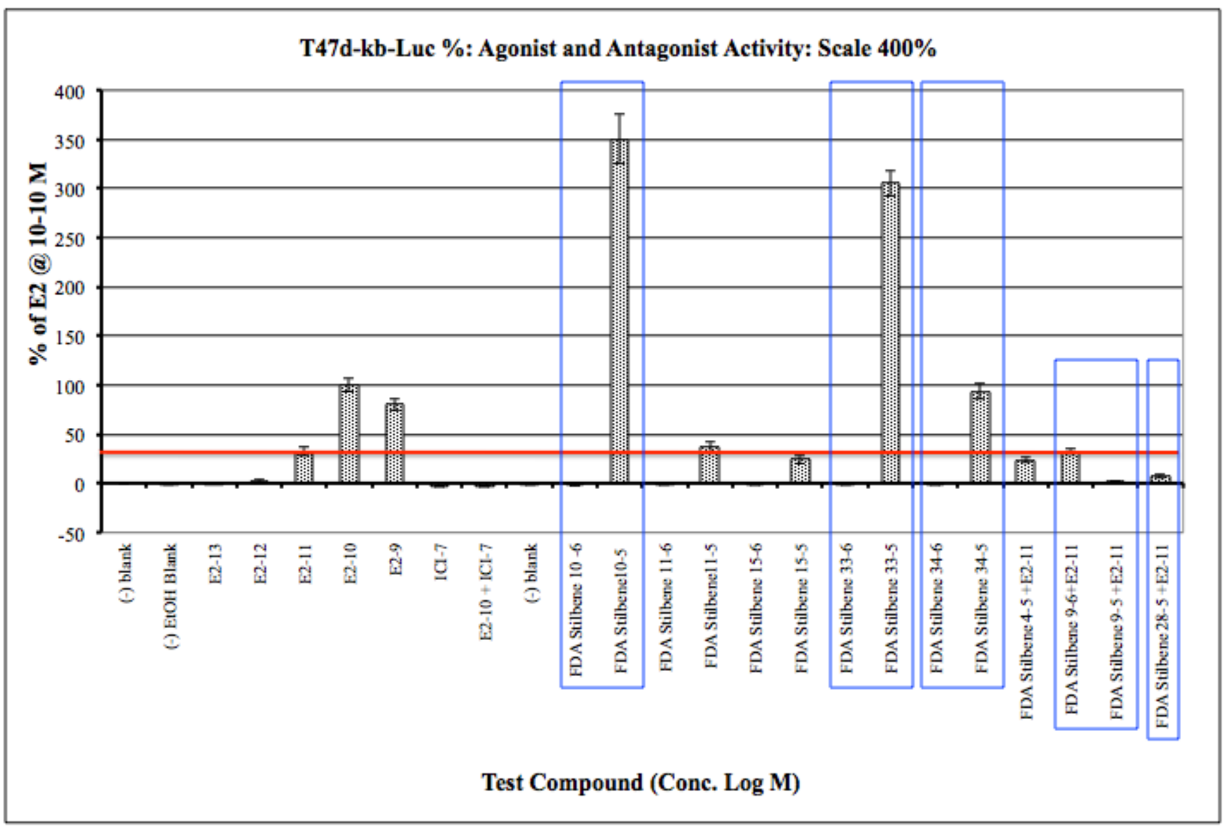


T47d-kb-Luc (A)
% of E2 @ 10-10 M
Test Compound (Conc. Log M)
120
110
100
90
80
70
60
50
40
30
20
10
0
-10
(-) blank
(-) EtOH Blank
E2-13
E2-12
E2-11
E2-10
E2-9
ICI-7
ICI-7/E2-10
(-) blank
FDA Stilbene 1 -5 M+E2-11
FDA Stilbene 2 -5 M+E2-11
FDA Stilbene 3 -5 M+E2-11
FDA Stilbene 4 -5 M+E2-11
FDA Stilbene 5 -5 M+E2-11
FDA Stilbene 6 -5 M+E2-11
FDA Stilbene 8 -5 M+E2-11
FDA Stilbene 9 -5 M+E2-11
FDA Stilbene 10 -5 M+E2-11
FDA Stilbene 11 -5 M+E2-11
FDA Stilbene 12 -5 M+E2-11
FDA Stilbene 13 -5 M+E2-11
FDA Stilbene 15 -5 M+E2-11
E2-11

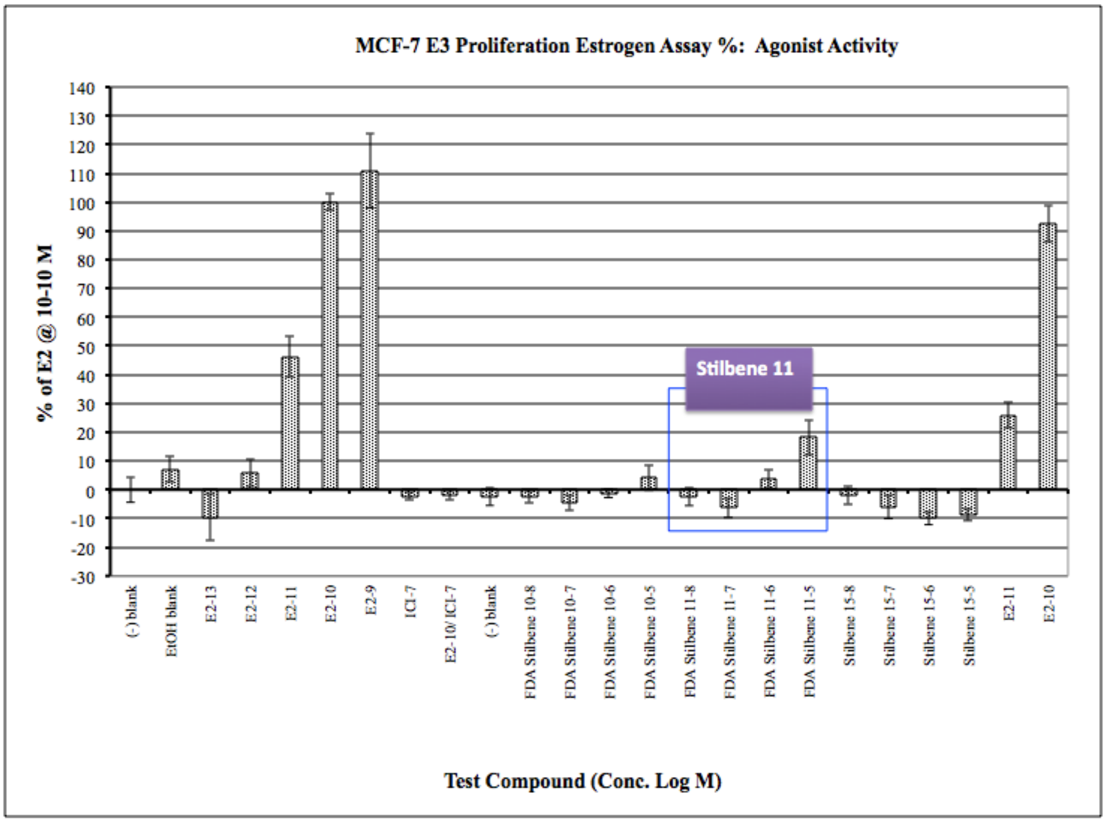


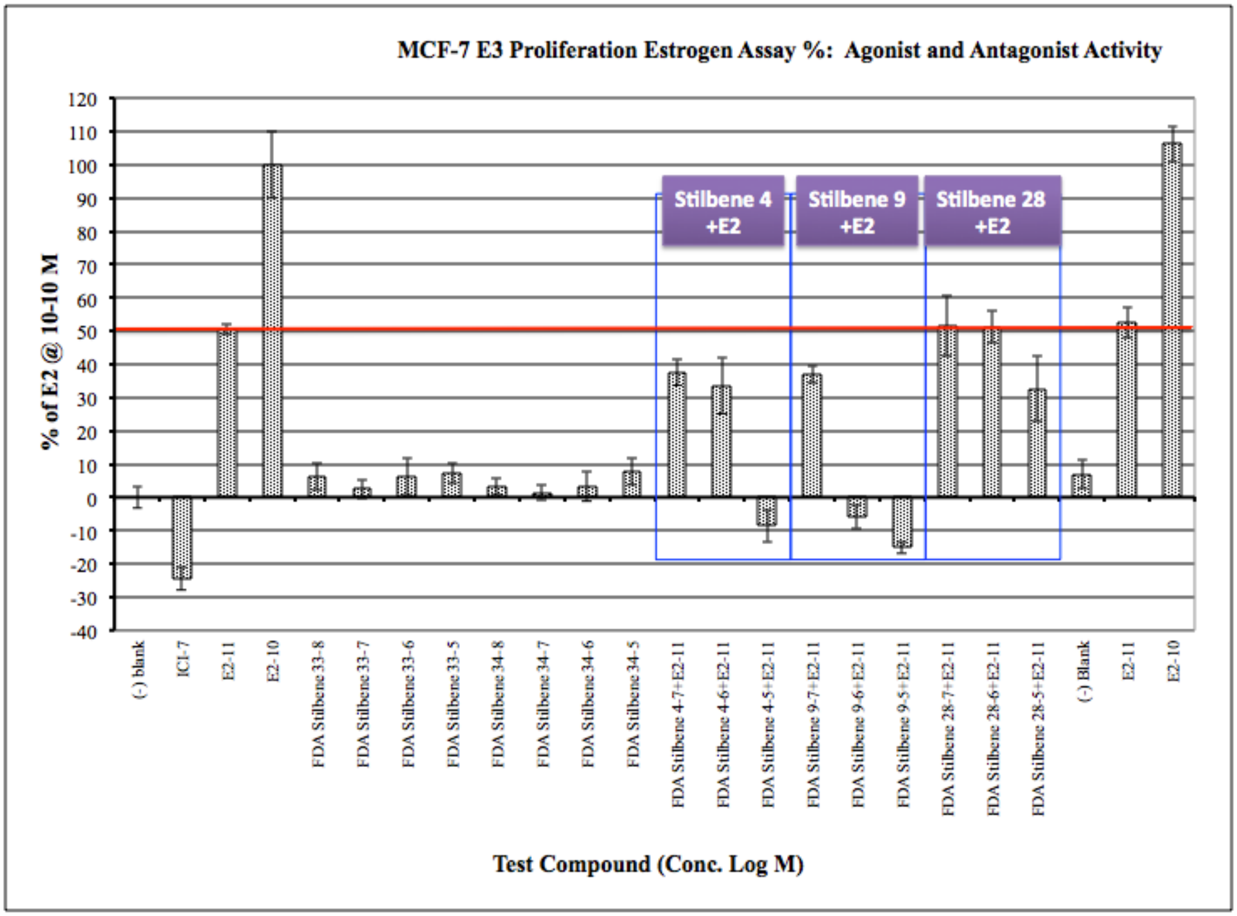
Year 3: Ms. Barbarini performed reporter gene assays for the 8 active stilbenes producing dose response data over the range of 10 nM to 10uM characterizing stilbenes 10, 11, 15, 33 and 34 as weak agonists active only at high concentrations. While weakly potent, stilbenes 10 and 33 where shown to induce exaggerated efficacy of 300% compared to the estradiol control. One the other hand, stilbenes 9 and 28 were shown to inhibit the estrogen receptor induced reporter gene more than 90% at high concentrations. Summary data is shown below.


T47d-kb-Luc %: Agonist and Antagonist Activity: Scale 100%
% of E2 @ 10-10 M
110
100
90
80
70
60
50
40
30
20
10
0
-10
(-) blank
(-) EtOH Blank
E2-13
E2-12
E2-11
E2-10
E2-9
ICI-7
E2-10 + ICI-7
(-) blank
FDA Stilbene 10 -6
FDA Stilbene10-5
FDA Stilbene 11-6
FDA Stilbene11-5
FDA Stilbene 15-6
FDA Stilbene 15-5
FDA Stilbene 33-6
FDA Stilbene 33-5
FDA Stilbene 34-6
FDA Stilbene 34-5
FDA Stilbene 4-5 +E2-11
FDA Stilbene 9-6+E2-11
FDA Stilbene 9-5 +E2-11
FDA Stilbene 28-5 +E2-11
Test Compound (Conc. Log M)


T47d-kb-Luc %: Agonist and Antagonist Activity: Scale 400%
% of E2 @ 10-10 M
400
350
300
250
200
150
100
50
0
-50
(-) blank
(-) EtOH Blank
E2-13
E2-12
E2-11
E2-10
E2-9
ICI-7
E2-10 + ICI-7
(-) blank
FDA Stilbene 10 -6
FDA Stilbene10-5
FDA Stilbene 11-6
FDA Stilbene11-5
FDA Stilbene 15-6
FDA Stilbene 15-5
FDA Stilbene 33-6
FDA Stilbene 33-5
FDA Stilbene 34-6
FDA Stilbene 34-5
FDA Stilbene 4-5 +E2-11
FDA Stilbene 9-6+E2-11
FDA Stilbene 9-5 +E2-11
FDA Stilbene 28-5 +E2-11
Test Compound (Conc. Log M)

To further characterize the potential for the 8 “active” stilbenes to induce agonist or antagonist estrogen activity, the MCF-7 proliferation assay was used. These determinations attempt to show efficacy in terms of a natural cellular response: proliferation. Comparison of the reporter gene and proliferation results has shown that while the reporter gene assay is a very sensitive screen for ER activity, the impact on cell proliferation may be more muted. Samples data is shown below.





From these *in vitro* characterizations of the 28 stilbenes, 8 have shown to have significant activity in the reporter gene and cell proliferation systems. In year 4, we will focus on these 8 compounds and determine ER alpha and beta binding activity (Task 6B) and include only these compounds in Tasks 6d-h. We will then focus on the most active of the 8 compounds in Task 7 (Stilbenes 4, 9, 11).

6d - Perform ER alpha/beta selective reporter gene assays of test compounds (Months 12-20)

6e - Evaluate test compounds in estrogen/breast cancer PCR array (Months 16-30)

6f - Data analysis of PCR array data (Months 28-33)

6g - Evaluate coactivator ER interactions with test compounds bound using LanthaScreen™ TR-FRET ER alpha/beta Coactivator Assay (Months 16-35)

6h - Perform genome wide shRNA library screen coupled with gene expression arrays of sensitive cells to identify drug targets, drug sensitizers, and drug-resistance pathways (Months 18-30)

Tasks 6d-6h have not been initiated.

Task 7 - *In vivo* validation of estrogen activity. (Months 33-48)

7a - Test compounds for uterotrophic activity in mice (months 33-37)

7b - Examine antiestrogenic capacity of test compounds in uterotrophic assays (months 34-38).

7c - Evaluate activity of test compounds on breast cancer xenografts (months 38-48).

Task 7 has not been initiated.

<u>Sridhar/Jones/Stevens Subproject (Identification of a New Class of Tyrosine Kinase Inhibitors)</u>

The research accomplishments of this subproject include the following:

Task 1- Hire research associate to assist in project. (Month 1)

Dr. Jayalakshmi was hired as a research associate. Her expertise in organic chemistry and skills in molecular modeling made her an ideal fit for this project. In August of 2012, she joined the Xavier University Chemistry Department in a new position as a tenure-track Assistant Professor. In her new capacity, she co-directs this subproject with Dr. Cheryl Stevens who has left Xavier for a position as the Dean of the College of Science and Engineering at Western Kentucky University. Dr. Stevens and Dr. Sridhar have agreed to continue collaborating on this subproject with the goal of developing Dr. Sridhar into a prolific and well-trained cancer researcher.

Task 2- Identify student to assist in project. (Month 3)

Year 1: Due to Dr. Stevens leaving Xavier University in January 2012, no students were hired on this project in Year 1.

Year 2: Two students worked on the Project in Year 2 (Thuy-Linh Nguyen, and Jasmine Thompson).

Year 3: Four students have been working on the Project in Year 3 (Nancy Pham, Phan Tram, Jasmine Geathers, and Don Q. Nguyen).

Task 3- Identify novel small molecules related to quinazoline, tyrphostin, emodin, and dasatinib that inhibit HER2 activity. (Months 1-24)

3a - Identify detailed pharmacophore and determine geometric, electronic, and lipophilic characteristics required for tyrosine kinase inhibition (Months 1-12)

HER2 is a growth factor receptor protein belonging to the tyrosine kinase receptor family. HER2 is overexpressed in 25-30% of breast cancer patients and its overexpression has been detected in several other cancers including prostate cancer, ovarian cancer, lung cancer, mammary carcinoma, liver tumors, and colorectal cancers. Trastuzumab is a humanized antibody targeting the extracellular domain of HER2 that is currently being used clinically. Among the many tyrosine kinase inhibitors developed so far, only Lapatinib is in clinical use. Several other HER2 kinase inhibitors are in various stages of clinical trials.

The splice variant HER2Δ16 isoform lacking exon 16 preceding the transmembrane domain shows low sensitivity to Trastuzumab. This makes the development of a HER2 kinase inhibitor a more reasonable approach. Castiglioni, et al.[1] have shown that emodin (1,3,8-trihydroxy-6-methyl-anthraquinone) was the only drug that inhibited the therapeutically resistant oncogenic HER2 isoform, HER2Δ16. Based on these reports, emodin was chosen as the lead structure for development of HER2Δ16 inhibitors. Emodin and Iressa were first docked onto the HER2 homology model to study their binding modes with the help of MOE docking tools. Iressa did not bind to the hinge region residues of the protein. However, emodin did bind to the hinge region of the protein and three binding modes were identified (Figure 1). Based on the orientation of emodin in the binding pocket of the protein, residues that could be targeted for developing a good inhibitor were identified. These were Thr95, Gln96, Met98, Asp160, Lys50, Glu67, Thr159 (Figure 1).

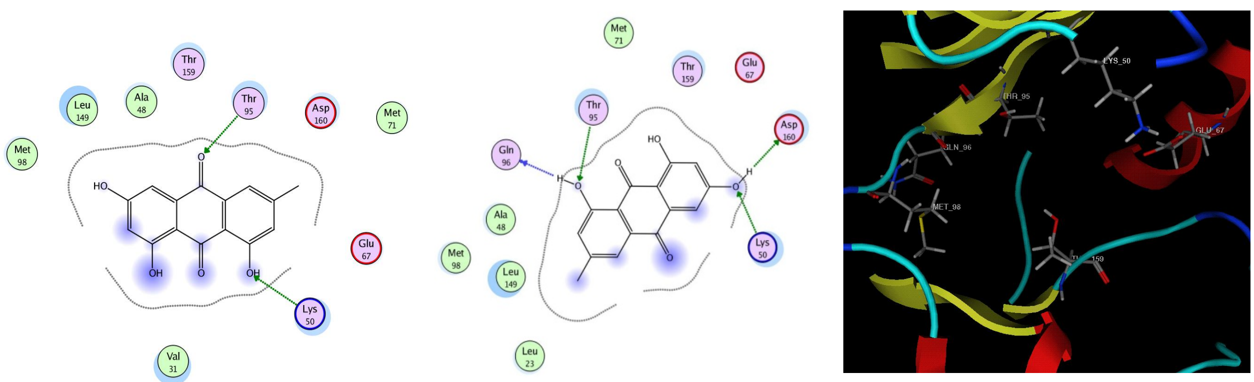
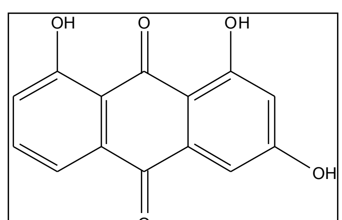


Figure 1. Binding modes of emodin onto HER2 protein homology model and a picture of the binding pocket with the potential target residues depicted in stick mode.

3b - Identify new compounds to be tested for tyrosine kinase inhibition with conformationally flexible searches of compound databases using detailed pharmacophore and CoMFA QSAR results. (Months 9-24).

Year 1: 1,3,8-trihydroxyanthraquinone was taken as the pharmacophore for a UNITY 2D-search of all the databases



1,3,8-trihydroxyanthraquinone

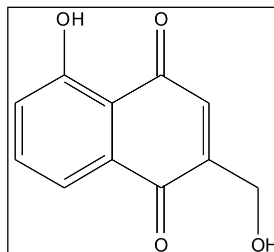
available to us. Hits were obtained from ACD (10 hits), TSCA (3 hits), and NCI databases (39 hits). NCI database hits overlapped significantly with the compounds contained in ACD hits.

Table 1: High-throughput screening of database hits against MCF7-HER2Δ16 cell line.

Compound	**% of E2 at 10^{-5}M**
AG-650/41069241	91.27
AG-650/41069319	11.11
AG-650/41069355	98.16
AP-782/41885488	0.26
AQ-776/42801622	87.79
AE-508/36399063	95.19
AP-782/21243033	96.81
AN-967/15488023	95.16
AG-650/41069356	0
AE-848/13198350	100
NSC322354	0.04
NSC227279	0.1
NSC109351	52.35
NSC202069	95.71
NSC299384	74.26
NSC309875	88.56
NSC309876	89.11
NSC310337	82.17
NSC310338	94.35

NSC319437	82.2
NSC367088	88.97
NSC379572	96.6
NSC379866	96.79
NSC93419	18.04
NSC7794	100.94
NSC138557	80.5
NSC204855	9.11
NSC251670	101.55

Year 2: Based on the initial high-throughput assay against MCF7-HER2Δ16 cell lines (Table 1), 13 compounds were chosen for further analysis (Table 2). Structures of these compounds are shown in figure 2. Additionally 3D and 2D-databases are being compiled using the commercially available compounds from TIMTEC, LC laboratories, Maybridge, and Pubchem. Based on the docking studies (explained in Task 4a) of the active compounds 4, 5 and 13 from Table 2; the essential features required for the inhibitor pharmacophore has been reduced to 5-hydroxy-2-(hydroxymethyl)naphthalene-1,4-dione. Database searches are currently underway to find new compounds that satisfy this pharmacophore. The hits obtained from the database searches will be docked and common core structures will be identified as new lead molecules.

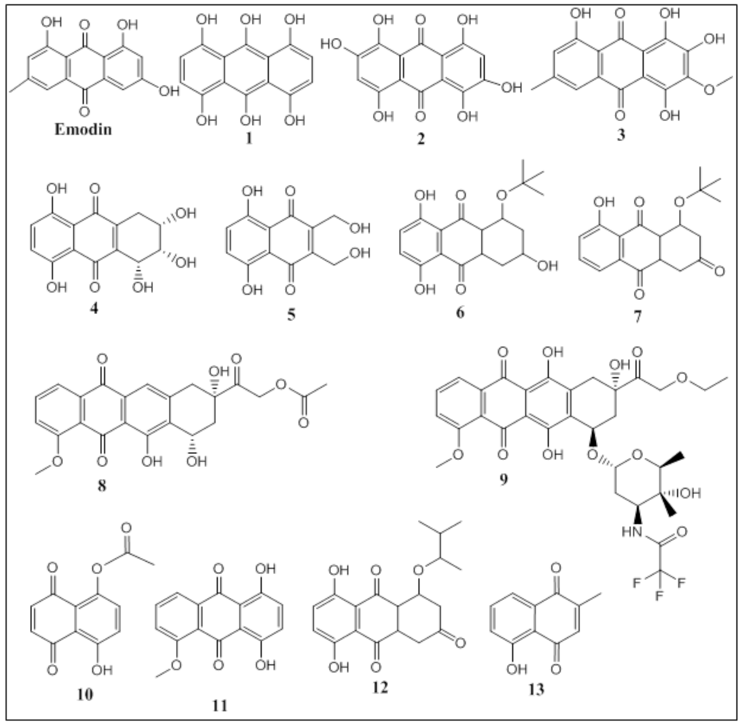


5-hydroxy-2-(hydroxymethyl) naphthalene-1,4-dione

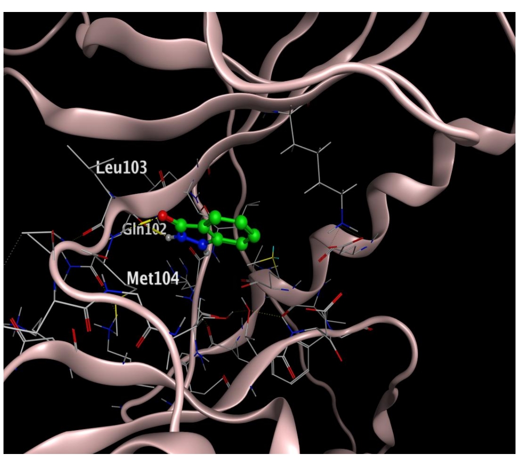
Table 2: The 13 compounds that were chosen for further analysis

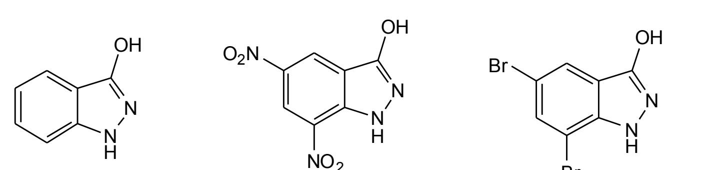
No.	Compound
1	NSC313**36**
2	NSC4011**45**
3	NSC2574**50**
4	NSC3223**54**
5	NSC2272**79**
6	AG-650/41069**129**
7	AG-650/41069**131**
8	AG-650/41069**241**
9	AG-650/41069**319**
10	AG-650/41069**356**
11	AG-650/41069**360**
12	AG-650/41069**378**
13	AP-782/41885**488**

Figure 2: Structures of the 13 compounds listed in table 2.



Year 3: During our database search using the pharmacophore designed earlier for novel core structures, 1H-indazol-3-ol (Indazolone) was found to dock well in the hinge region of the HER2 kinase. Two hydrogen bonds were formed between the indazolone and the HER2 residues Gln102 & Met104. Functionalization of the phenyl ring with suitable substituents can be achieved using well-known reactions (Figure 3). Hence this scaffold was chosen for further exploration. Three compounds were synthesized as initial lead structures (Figure 4).





1*H*-indazol-3-ol   5,7-dinitro-1*H*-indazol-3-ol   5,7-dibromo-1*H*-indazol-3-ol

Figure 4: Structures of the three 1H-indazol-3-ol synthesized.

Figure 3: Binding mode of 1H-indazol-3-ol (indazolone) to the ATP-binding region of HER2 kinase. The ligand is shown as ball and stick model.

Task 4- Explore the mechanism of HER2 tyrosine kinase inhibition. (Months 12-48)

4a - Dock proven and proposed TKIs into the tyrosine kinase ATP binding site using multiple poses, and score results. (Months 12-24)

Year 1: All of the hits described in **3b** were then docked onto the homology model of HER2 using MOE dock tools. The docking results were then studied manually. Binding of the molecule to one of the hinge region residues THR95, GLN96, MET98 was taken as a prerequisite. The number of ligand-protein hydrogen bond interactions, the extent of penetration of ligand into the pocket and the nature of ligand solvent exposure (hydrophobic/hydrophilic) were also considered.

Year 2: The three compounds that showed significant potency against the MCF-7 pcDNA, MCF7-HER2 and MCF-7 HER2Δ16 were subjected to docking studies onto the 3D-structure of HER2 kinase region (PDB ID: 3CRD.pdb) using MOE docking module and Surflex in SYBYL-X1.3. The consensus binding modes for these three compounds are shown in Figure 5. The docking modes of the compounds show that the phenolic group forms a hydrogen bond with the hinge region residue Gln799 and an additional hydrogen bond by the side chain hydroxyl group either with the invariable Lys753 for compound **4** or with Asp863 for compound **5**. Compound **13** made only one hydrogen bond with the protein which could account for its lower potency (refer to bioassay results in Task 4d). This led us to the optimal core structure of 5-hydroxy-2-(hydroxymethyl)naphthalene-1,4-dione as our lead structure.

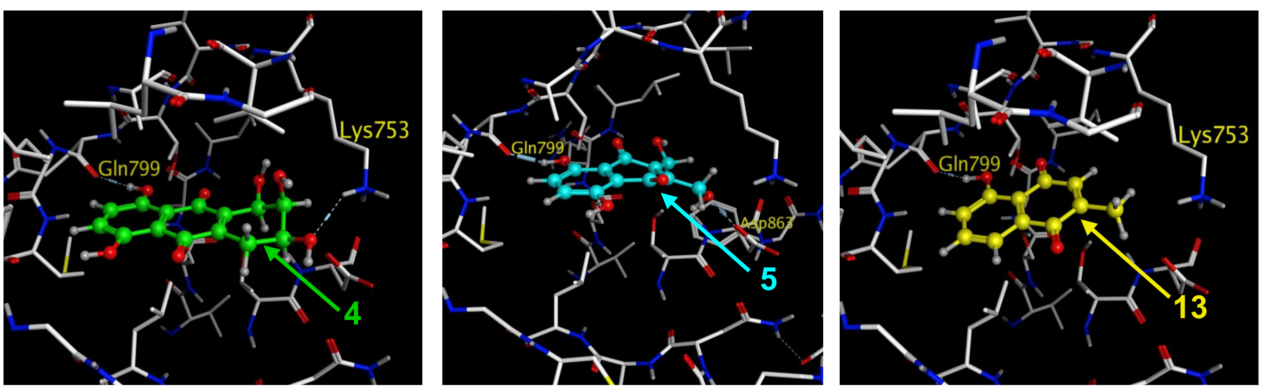
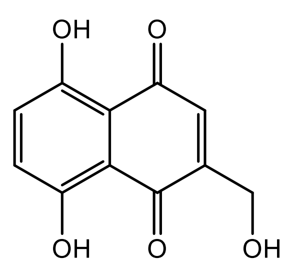
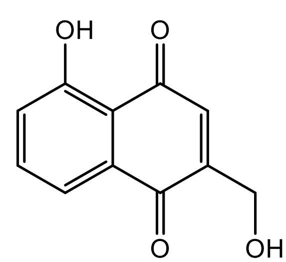


Figure 5: (A) **4**, (B) **5** and (C) **13** in the active site of HER2 kinase. The protein (PDB ID: 3CRD.pdb) residues are shown as stick models and the compounds are shown as ball & stick model.

4b - Optimize molecular structures to maximize ability of compounds to inhibit HER2. (Months 15-30)



LM1



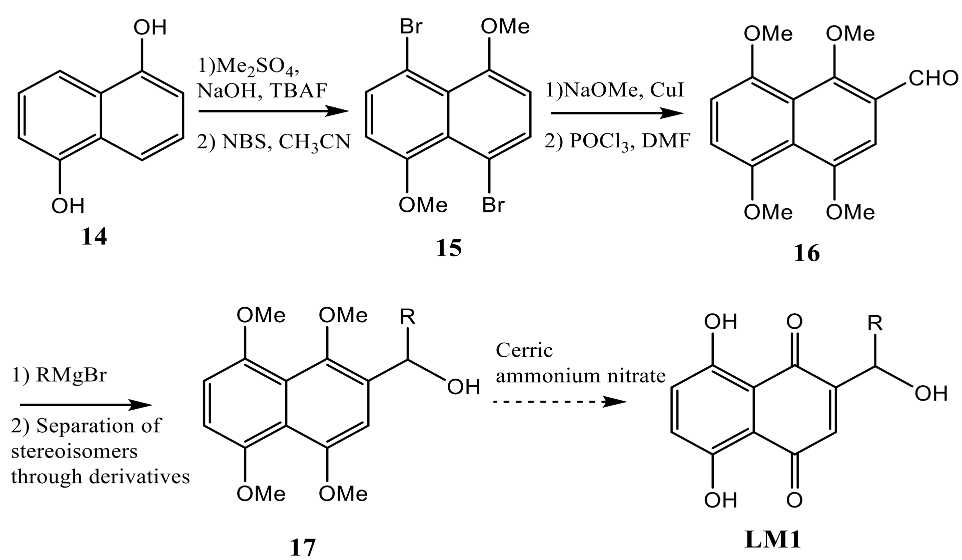
LM2

Year 1: Not initiated.

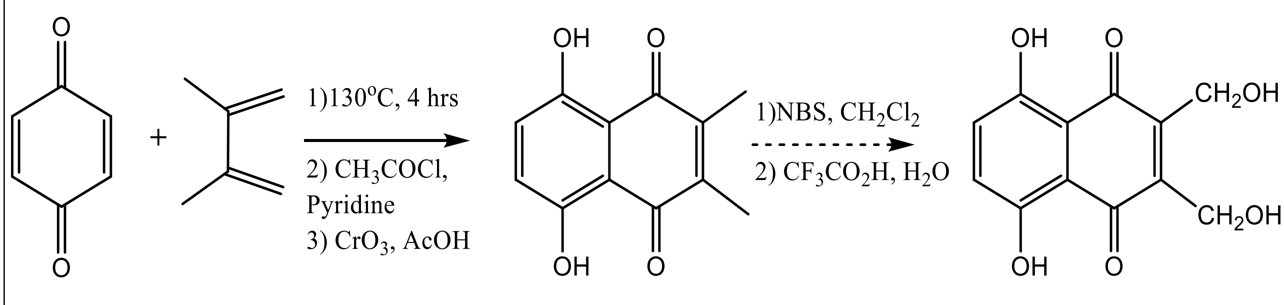
Year 2: From the bioassay results (Task 4d) and the docking studies it was clear that two core structures that can maintain the two hydrogen bonds shown by compound **5** can serve as the lead molecules **LM1** and **LM2**. The synthetic protocol for the lead molecule LM1 has been designed as given in the Scheme 1 involving many known reactions (Tanoue and Terada, *Bull. Chem. Soc. Jpn.,* (1988) **61**, 2039). The reactions until the formation of compound **17** have been standardized. Different Grignard reagents will be used to obtain variation in the R-group on compound **17**. The purification of compound **17** and its conversion to LM1 by oxidation with cerric ammonium nitrate is currently being pursued.

Scheme 2 provides an alternate route for the synthesis of the lead compound LM1 by using the Diels-Alder reaction (Paull *et al.*, *J. Med. Chem.*, (1976), **19**, 337). The reactions in this scheme for the formation of the dihydroxynaphthoquinone **18** have been standardized. Further reactions are presently being carried out. Once the final diol is obtained from compound **18**, further reactions will be performed to obtain derivatives with different functional groups on the side chains and with differing chain lengths.

Scheme 1:



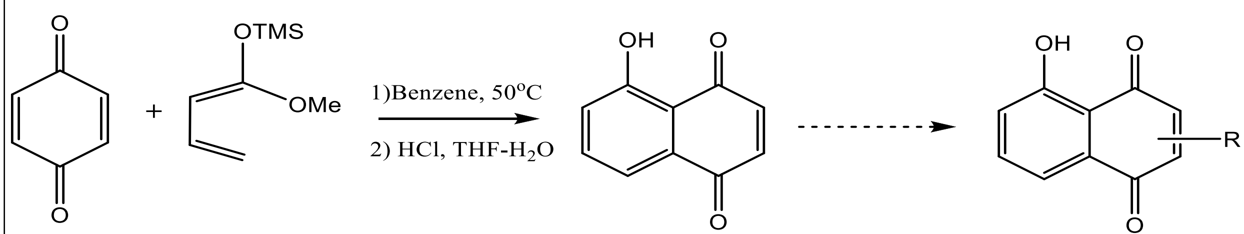
Scheme 2:



18 **LM1**

The synthesis of the lead molecule **LM2** will be pursued using Scheme 3 which is similar to Scheme 2 in that both the schemes involve the Diels-Alder reaction as the first step. The first reaction to compound **18** in Scheme 3 is presently being standardized. Upon standardization of the reactions, different derivatives of LM2 will be obtained by using suitably substituted starting materials and/or by derivatization of the

Scheme 3:



18 **LM2**

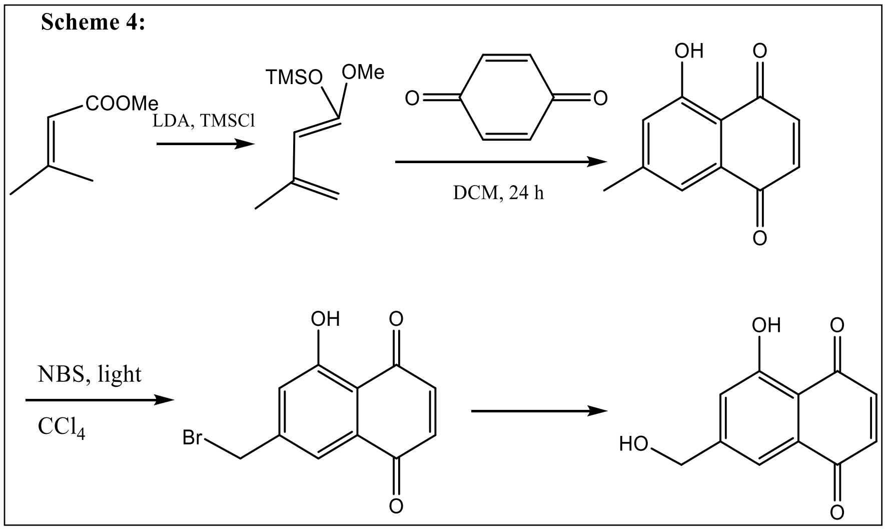
hydroxynaphthoquinone LM2.

Year 3: Toxicology studies on HeLa cells (given in section 4d) revealed that among the three compounds that showed good inhibition of the MCF7/HER2 andMCF7/HER2Δ16 cell lines, two of them drugs **4** and **5** were highly toxic. Toxicology studies for the quinones that showed inhibition activity were also subjected to toxicology studies on DU-145 cells. All of these studies revealed that compounds that contain the oxygen atom marked by the red circle (Figure 13) were highly toxic. Our strategy is to avoid toxicity and improve the potency of the drug molecules.

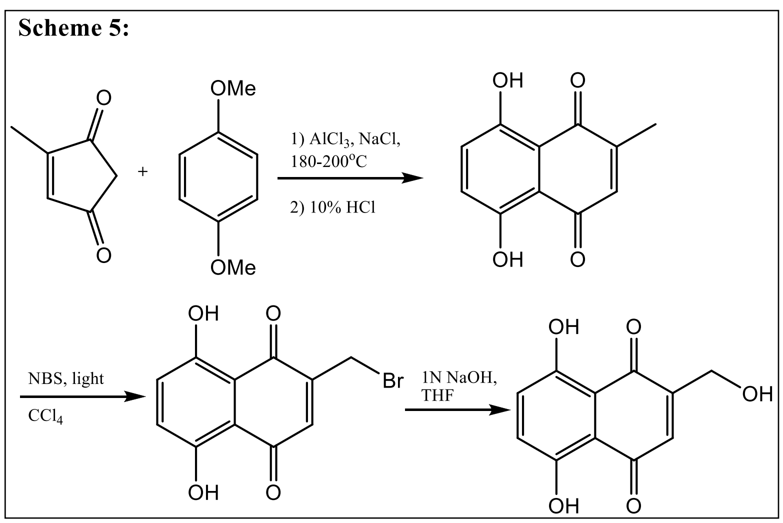
Hence our lead compound structure has been modified to target the residues Gln799 and Asp863. We have synthesized several derivatives of quinones LM1 (R = H-, CH_3-, CH_3CH_2-) using the Scheme 1 outlined earlier. The following schemes were employed for the syntheses of 5-hydroxy-7-methylnaphthalene-1,4-dione and 5,8-dihydroxy-2-methylnaphthalene-1,4-dione which were then converted to their bromomethyl derivatives and subsequently the alcohol derivatives (Scheme 4). These compounds will

be subjected to in-vitro inhibition assays against MCF7 cell lines the within the next couple of months to check on their activity and toxicity. These derivatives will be used for the synthesis of further analogs.

Scheme 4:



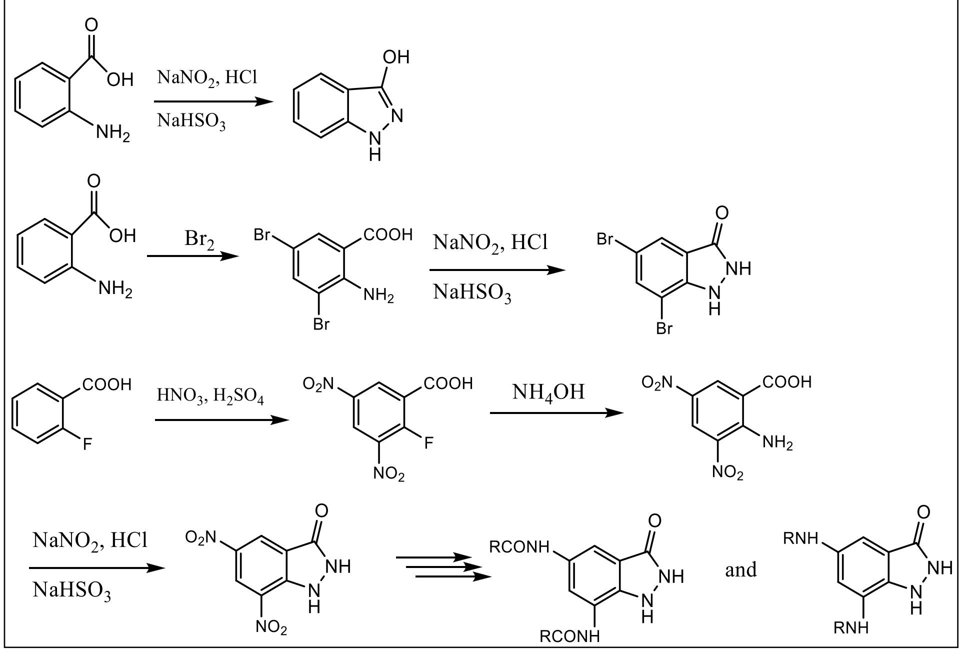
Scheme 5:



The synthesis of the indazolone core structures was initiated. Three starting molecules are being synthesized (Scheme 6). The dinitroindazolone can be used to prepared many different analogues as the nitro groups can be reduced and functionalized further using suitable substituents on them to give us diamines or diamides. The reduction of the nitro groups using H_2/Pd will be attempted next followed by acylation and alkylation to get the final compounds. The dibromoindazolone can be used to prepare many

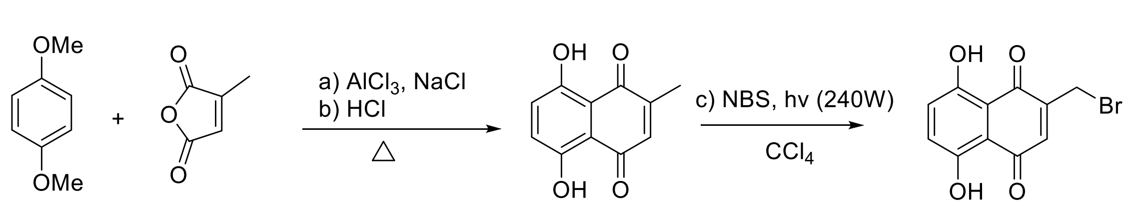
different derivatives using coupling reactions such as Suzuki coupling to provide us with alkyl and aryl substituents on the core indazolone moiety. The Suzuki coupling reaction is presently being standardized. Indazolone, dinitroindazolone and dibromoindazolone have been prepared and are presently being subjected to bioassay for inhibition of MCF7 cell lines.

Scheme 6: Synthesis of 1H-indazl-3-ol/indazolone series of compounds

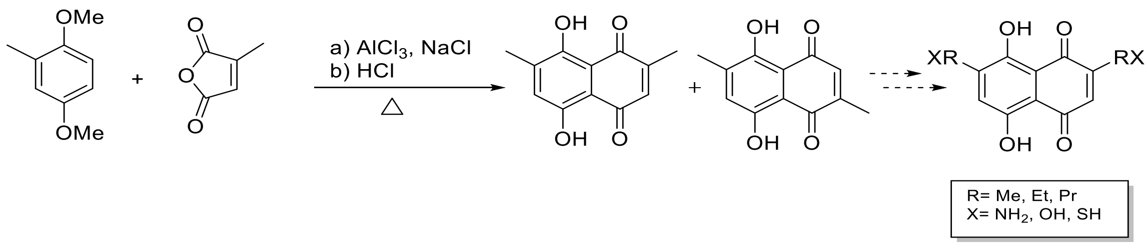


Year 4: Several compounds shown below were synthesized using the synthetic schemes given above. All of these compounds were then subjected to high-throughput screening for the growth inhibition of MCF7, MCF7-HER2Δ16 cell lines. Three other synthetic schemes (Schemes 7, 8 and 9) were standardized for the synthesis of new naphthoquinone derivatives.

Scheme 7



Scheme 8



Scheme 9

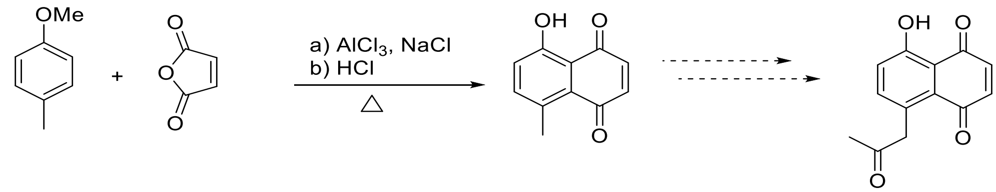
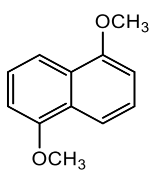
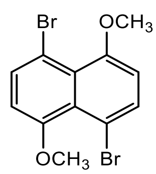


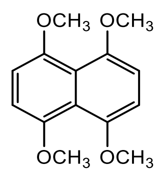
Figure 6: Structures of the compounds that were newly synthesized.



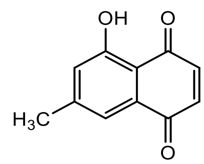
RLS 51



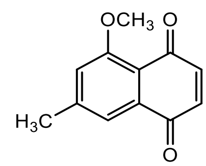
RLS 52



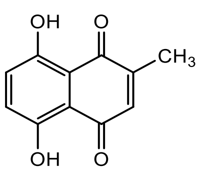
RLS 64



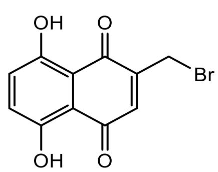
NG-1



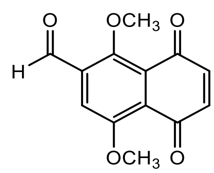
NG-2



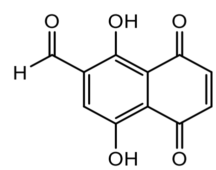
RLS 77



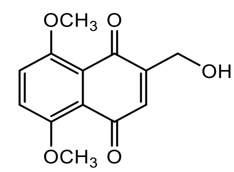
RLS 82



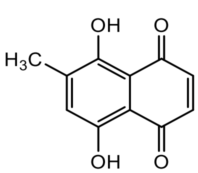
RLS 95



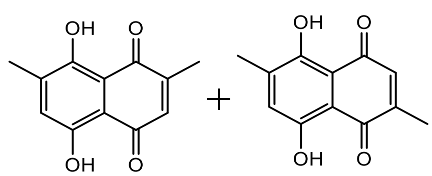
RLS 97



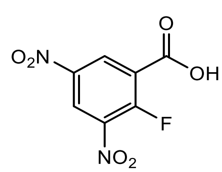
RLS 100-A



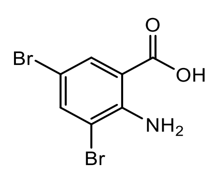
RLS 114



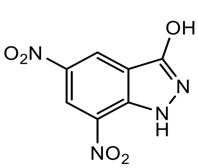
RLS 115



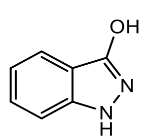
NP 82



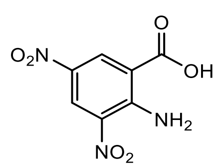
NP 64



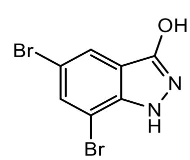
NP 78



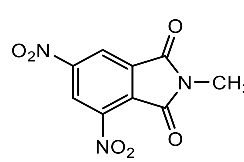
NP 45



NP 73



NP 66



RLS 73

4c - Attempt to identify alternate binding sites. (Months 18-30)

No progress yet.

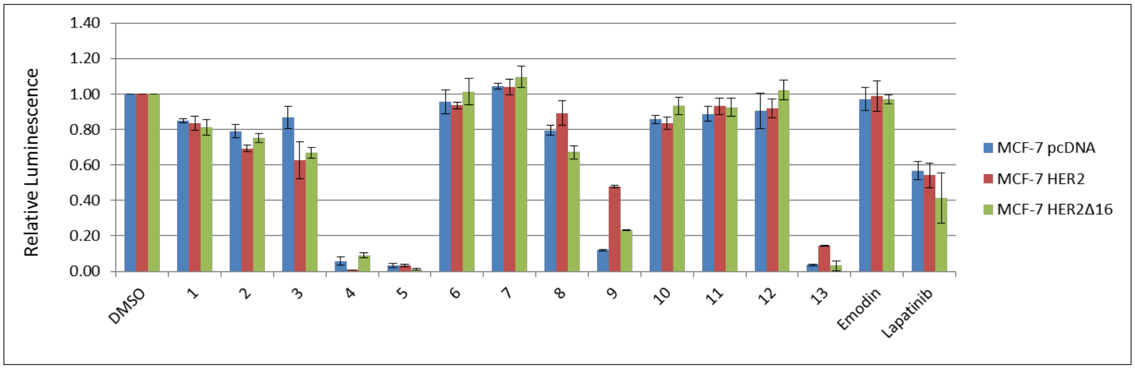
4d - Perform *in vitro* kinase inhibition and binding assays. (Months 18-48)

Year 1: A total of 28 compounds were procured from the Developmental Therapeutics Program NCI/NIH and Specs chemicals (Table 1). An initial high-throughput assay was performed to determine the inhibition of proliferation of MCF-7 cell line. The compounds that showed good inhibition activity were then subjected to *in-vitro* assay against HER2Δ16 cell line activity. Two compounds that showed low inhibition activity were included to confirm the activity profile of this set of compounds. Two of the tested compounds (NSC322354 and AG-650-41069319) showed low micro molar activity against the HER2Δ16 cell line (< 10 mM) (Table 1).

Year 2: We tested the ability of 13 small molecules (selected based on the high-throughput screening results given in Table 1) that are structurally similar to emodin to inhibit cell viability in MCF-7 breast cancer cell lines that express HER2, HER2Δ16 or empty vector. To do this we treated the cells for 48 hours with a concentration of 10μM for each set of drugs. After treatment, cell viability was tested using the CellTiter-Glo Assay (Promega) (Figure 6A). Of the 13 compounds, we found three that suppressed cell viability potently in all three cell lines (NSC322354 (Drug 4), NSC227279 (Drug 5), and AP-782/41885488 (Drug 13)). After 48 hours, lapatinib decreased cell viability by <50% while the three test drugs decreased cell viability by >90% at the same concentration (Figure 6B).

To determine the ability of the drug treatments to inhibit the activation of receptor tyrosine kinase HER2, Western blots were performed to detect total phosphorylated protein after treating each cell line with the three most potent drugs or lapatinib at 10μM for 2 hours. As expected, lapatinib dramatically decreases HER2 activating phosphorylation at auto-phosphorylation site Y1248 respectively in each of the cell lines. These sites were suppressed to the same extent in the cell lines after exposure to each of the test drugs, indicating that, like lapatinib, activation of both receptors was repressed by the test compounds effectively and quickly. We also tested phosphorylation of HER2 after exposure to one of the compounds (AP-650/41069356 (Drug 10)) that we found to have no effect on cell viability. This drug had no effect on the phosphorylation status of HER2, suggesting that the ability of the drugs to inhibit these receptor tyrosine kinases is critical to their ability to induce cell death.

A:



B:

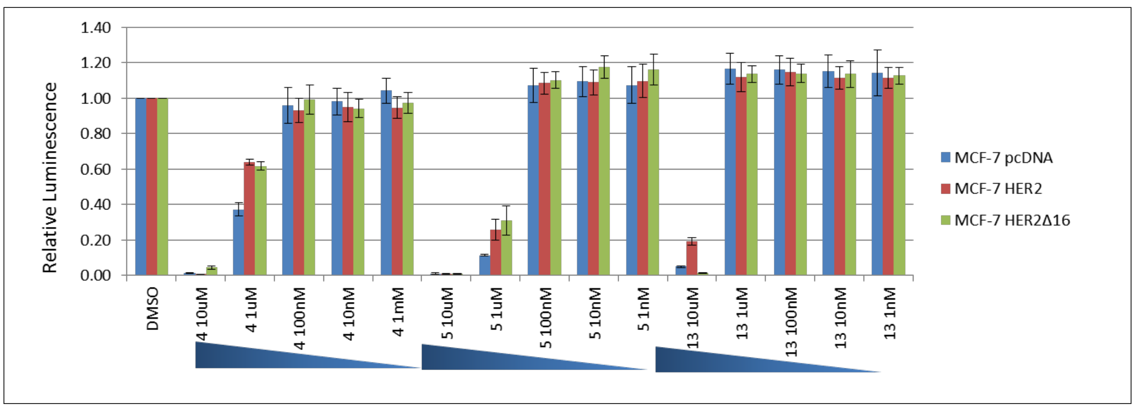


Figure 7: Inhibition of MCF7-pcDNA, MCF7-HER2 and MCF7-HER2Δ16 cell lines (A) High-throughput assay of emodin and compounds 1 – 13, (B) Inhibition assay of compounds **4**, **5** and **13** at different concentrations.

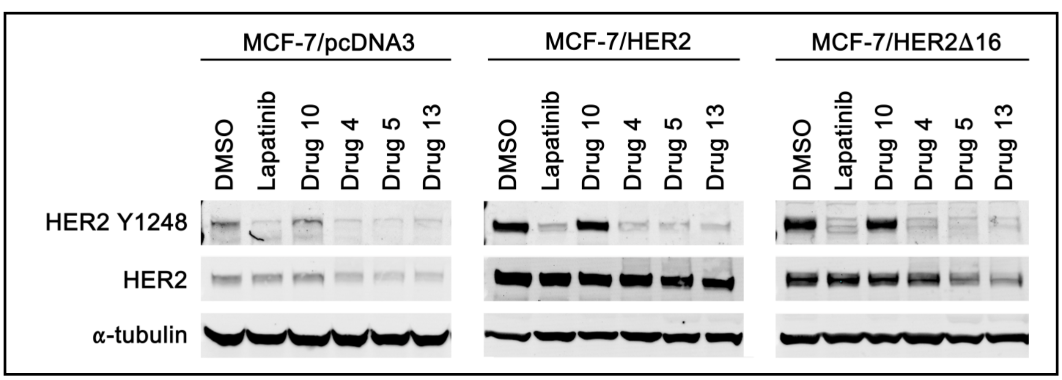


Figure 8: Western blot analysis of autophosphorylation at the HER2 residue Y1248. Compounds **4, 5** and **13** inhibit the phosphorylation at Y1248 in MCF7/pcDNA, MCF/HER2 and MCF7/HER216. Compound 10 which did not show notable inhibition in the high-through put assay shows significant phosphorylation at Y1248 in all of the three cell lines.

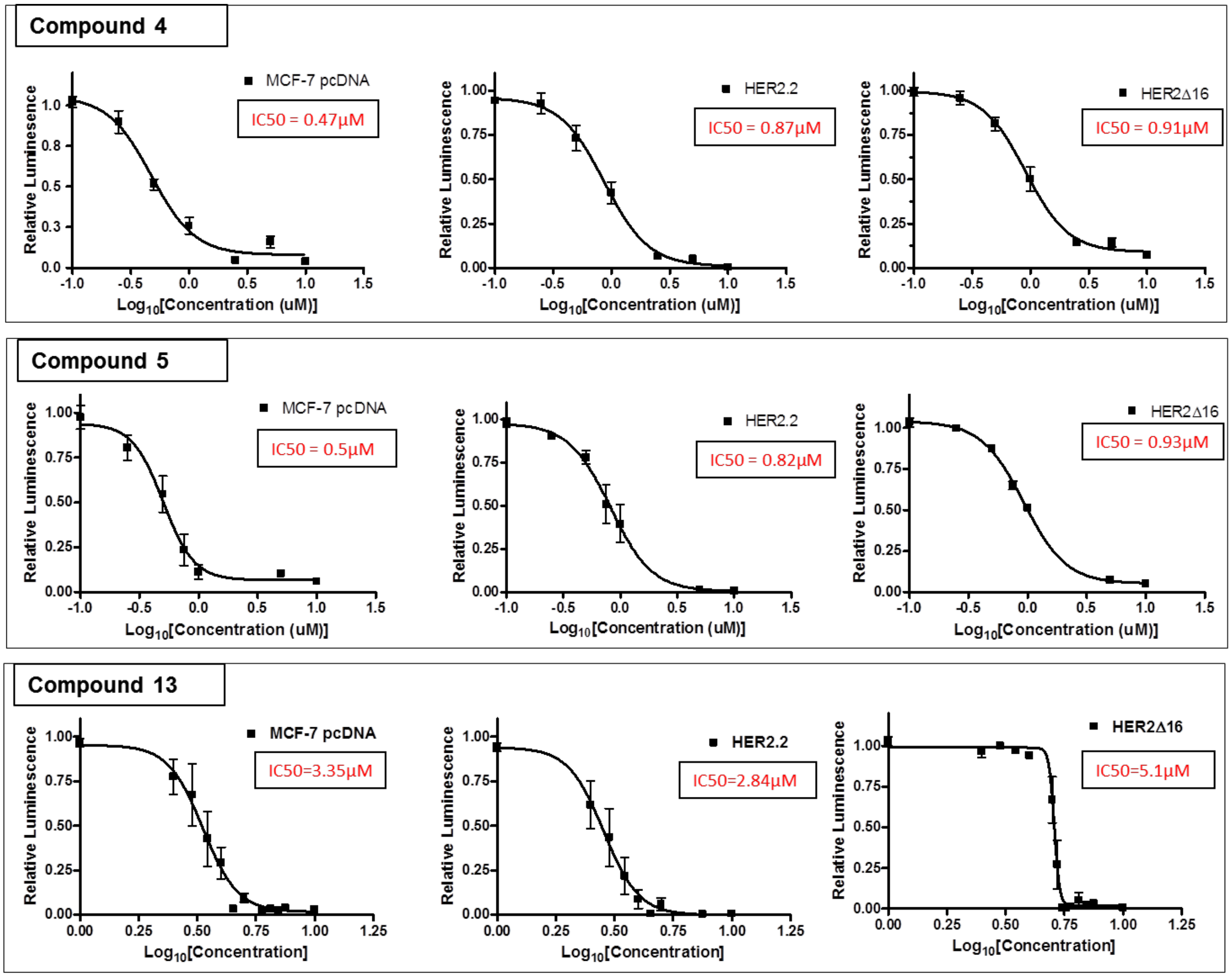


Figure 9: Inhibition of MCF-7/pcDNA, MCF-7/HER2 and MCF-7/HER2Δ16 cells by compounds **4, 5** and **13**.

Finally, we measured the IC_{50} of each drug by treating each of the cell lines with different drug concentrations for 48 hours then using the CellTiter-Glo Assay to detect cell viability (Figure 8). Cells overexpressing HER2Δ16 showed resistance to lapatinib (IC_{50} = 19.22µM) compared to wildtype HER2 overexpression (IC50 = 15.79µM). All three of the test drugs had a low µM IC_{50} for each of the cell lines. We found that the two most effective drugs at inhibiting cell viability (NSC322354 (Drug 4), NSC227279 (Drug5)) also had similar IC_{50} concentrations for both the HER2 (0.87µM, 0.82µM) and HER2Δ16 (0.91µM, 0.93µM) overexpression cell lines. With IC_{50} values of <1µM for HER2Δ16 cells, these two drugs are also more effective at inducing cell death compared to lapatinib with an IC_{50} >10µM. These results indicate the potential of either of these drugs to effectively inhibit HER2Δ16 action and thus combat the drug resistance seen in HER2Δ16 expressing tumors.

Year 3: Both EGFR and HER2 are members of the EGFR-family of receptor tyrosine kinases. All ligand activated or constitutively active members of this family have the

ability to dimerize with and transphosphorylate any other member of the family. In these specific cell lines, overexpression of HER2 or HER2Δ16 results in constitutive activation of the receptor. The constitutively active receptors heterodimerize with and transphosphorylate coexpressed EGFR. To determine the ability of the drug treatments to inhibit the activation of receptor tyrosine kinase EGFR, Western blots were performed to detect total phosphorylated protein after treating each cell line with the three most potent drugs or lapatinib at 10μM for 2 hours (Figure 9). The parental MCF-7 cell line lacks significant levels of HER2 phosphorylated activation; therefore HER2 fails to dimerize with and transphosphorylate EGFR. This explains why EGFR also remains unphosphorylated in parental MCF-7 cell line. As expected, lapatinib dramatically decreases EGFR activating phosphorylation at auto-phosphorylation site Y1068 respectively in each of the cell lines. These sites were suppressed to the same extent in the cell lines after exposure to each of the test drugs, indicating that, like lapatinib, activation of both receptors was repressed by the test compounds effectively and quickly. We also tested phosphorylation of EGFR after exposure to one of the compounds (AP-650/41069356 (Drug 10)) that we found to have no effect on cell viability. This drug had no effect on the phosphorylation status of EGFR, suggesting that the ability of the drugs to inhibit these receptor tyrosine kinases is critical to their ability to induce cell death.

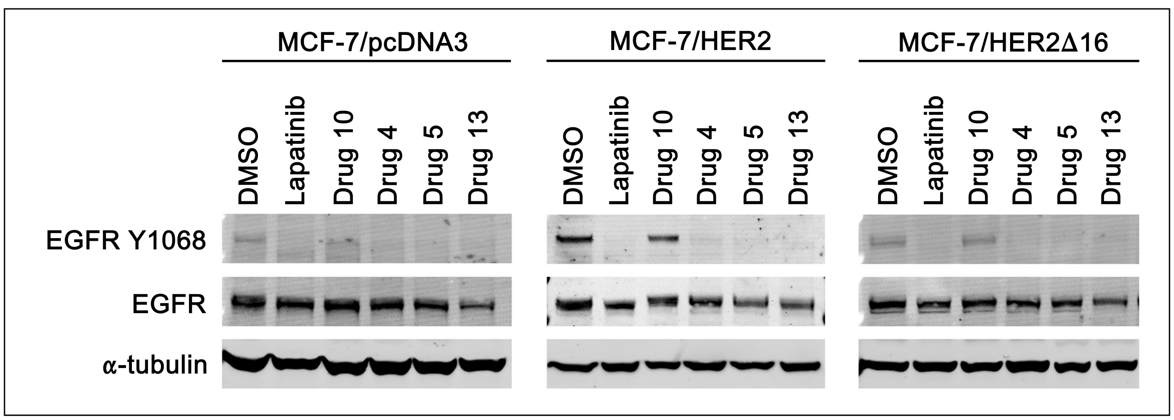


Figure 10: Western blot analysis of autophosphorylation at the EGFR residue Y1068. Compounds **4, 5** and **13** inhibit the phosphorylation at Y1068 in MCF7/pcDNA, MCF/HER2 and MCF7/HER216. Compound 10 which did not show notable inhibition in the high-through put assay shows significant phosphorylation at Y1068 in all of the three cell lines.

We tested the ability of the compounds to inhibit HER2 (Figure 10) and EGFR (Figure 11) kinase activity directly by using the ADP-Glo in vitro kinase assay kit from Promega, according to the manufacturer's instructions Briefly, 11ng of purified HER2/EGFR kinase was incubated with varying concentrations of each inhibitor in the presence of

10μM ATP to establish an inhibition curve for each. The data were fit to a logistic sigmoid function using Origin software, which calculated IC50 values for each drug. The well-known HER2 and EGFR dual inhibitor lapatinib had an IC50 of 0.05μM and 0.027μM respectively. The other three drugs had IC50's for HER2 and EGFR kinase inhibition as follows: compound 4 (10.8μM and 13μM), compound 5 (11.4μM and 15μM), compound 13 (30.8μM and 2μM), respectively.

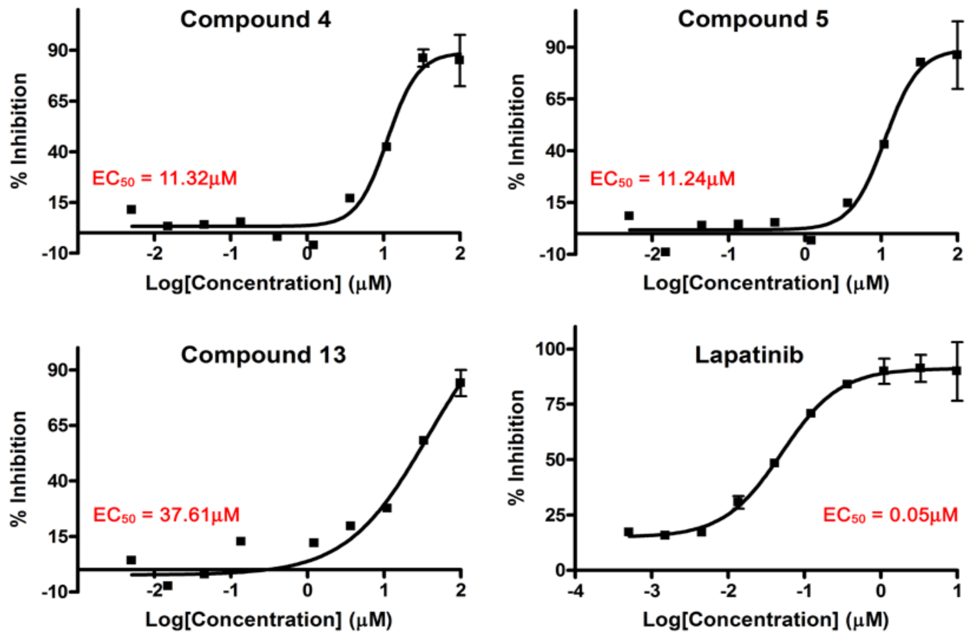


Figure 11: Dose response curves for inhibition of HER2 kinase by compounds **4**, **5**, **13** and lapatinib.

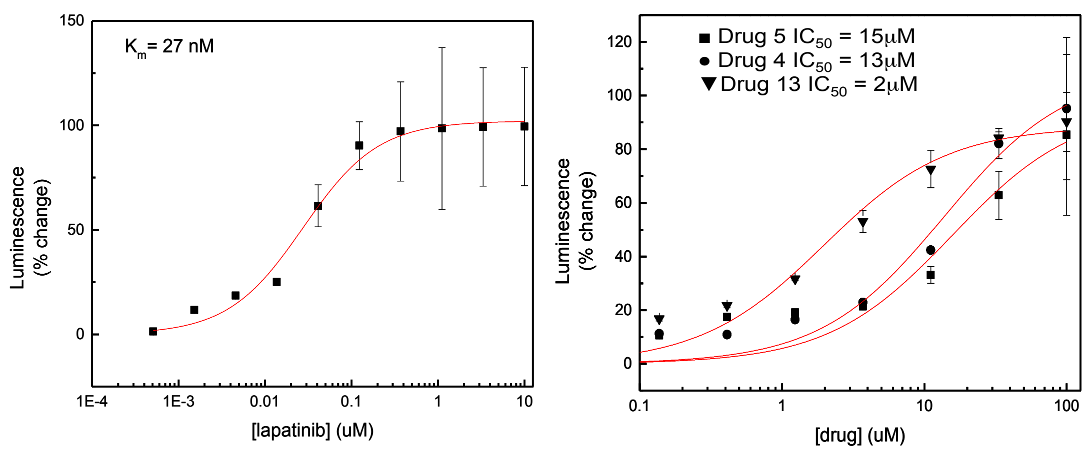


Figure 12: Dose response curves for inhibition of EGFR kinase by compounds **4**, **5**, **13** and lapatinib.

Toxicology Studies:

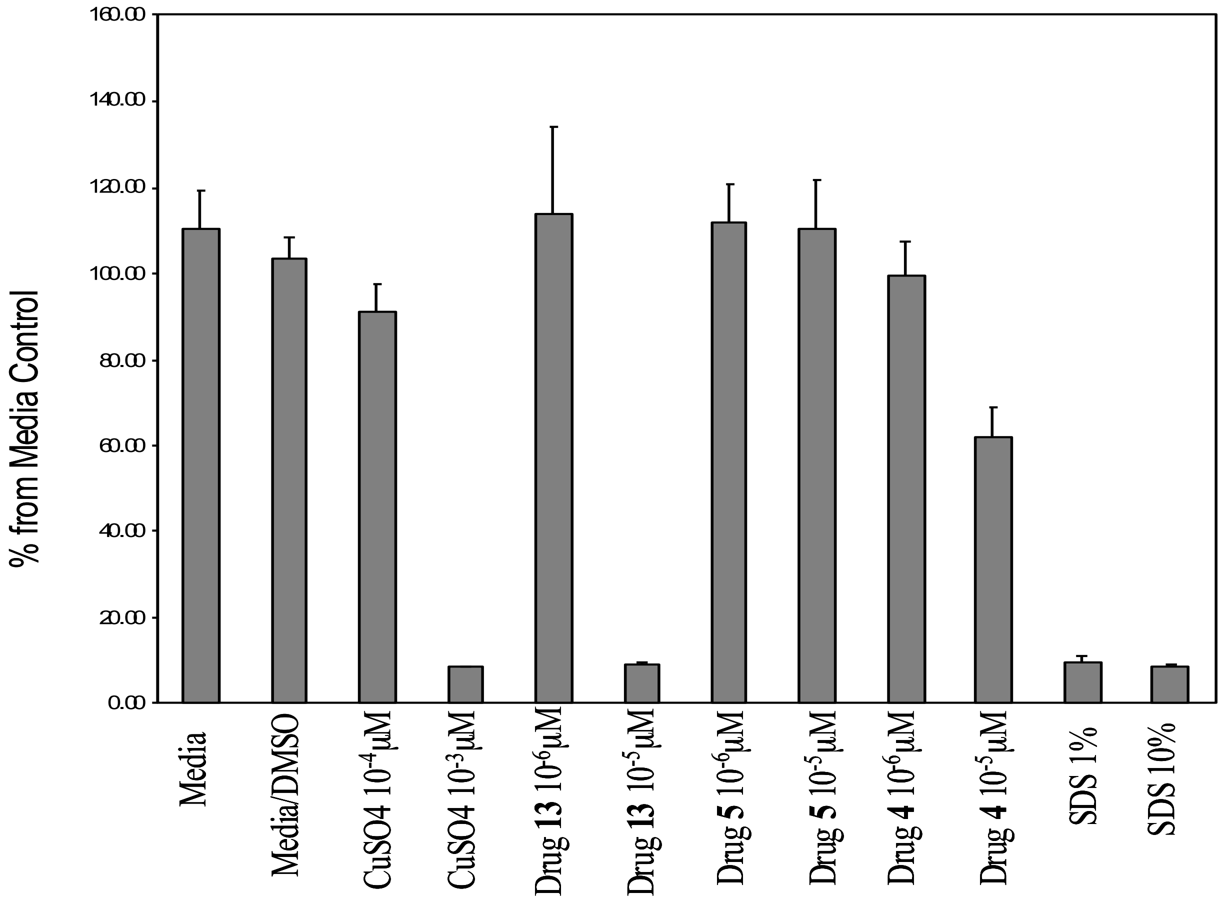


Figure 13: HeLa Alamar Blue cell viability assay for the compounds **4**, **5** and **13**.

HeLa cells were seeded in a 96-well plate at 0.9x105 cell/ml. The next day the media is replaced with 0.2% FBS media for 72 hours. At this point, an Alamar Blue assay is performed to calculate cell number per well. Drug is then added and the cells are counted again after an additional 72 hours. The ratio between the number of cells before drug and after incubation with drug is then calculated. The results indicated that compounds **4** and **13** were toxic at a concentration of 10^{-6} μM. Only compound **5** did not show any toxicity (Figure 12). Considering the toxicology assay results on HeLa cells and on the DU-145 cells (Task 5, Year 3), we could see compounds containing the oxygen atom marked with a 'red circle' were toxic (Figure 14). The hydroxyl group marked with the 'blue circle' seemed sufficient to maintain the same inhibition potency without toxicity. Hence we

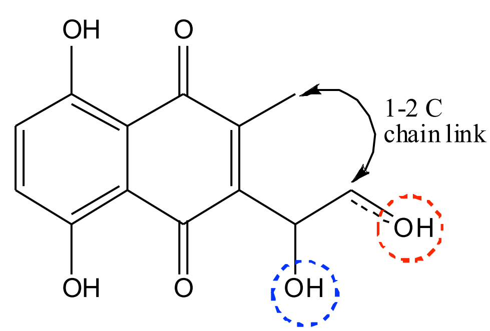


Figure 14: Essential structural features for the lead structure based on bioactivity and toxicity.

decided to pursue the synthesis of compounds containing only the 5,8-dihydroxy-2-(hydroxymethyl)naphthalene-1,4-dione moiety in them.

Year 4: The newly synthesized compounds (Figure 2.1) were subjected to high-throughput screening for growth inhibition of MCF7 pcDNA3, MCF7 HER2 and MCF7/HER2Δ16 cell lines (Figure 15).

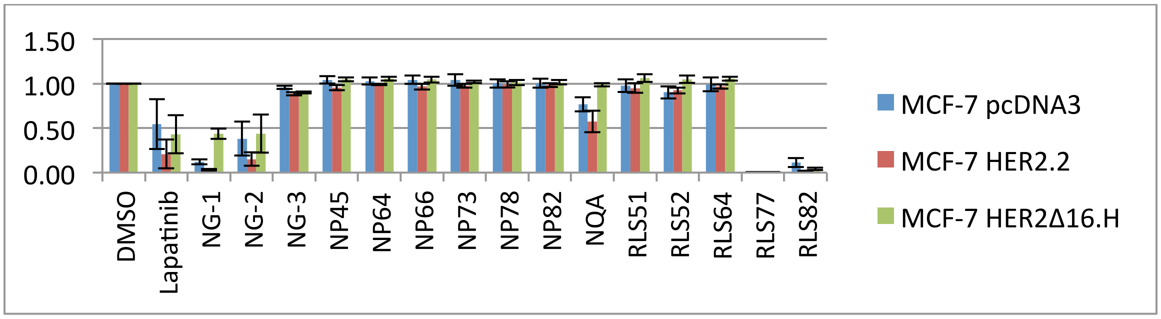


Figure 15: Inhibition of MCF7-pcDNA, MCF7-HER2 and MCF7-HER2Δ16 cell lines (A) High-throughput assay of the newly synthesized compounds given in Figure 2.1

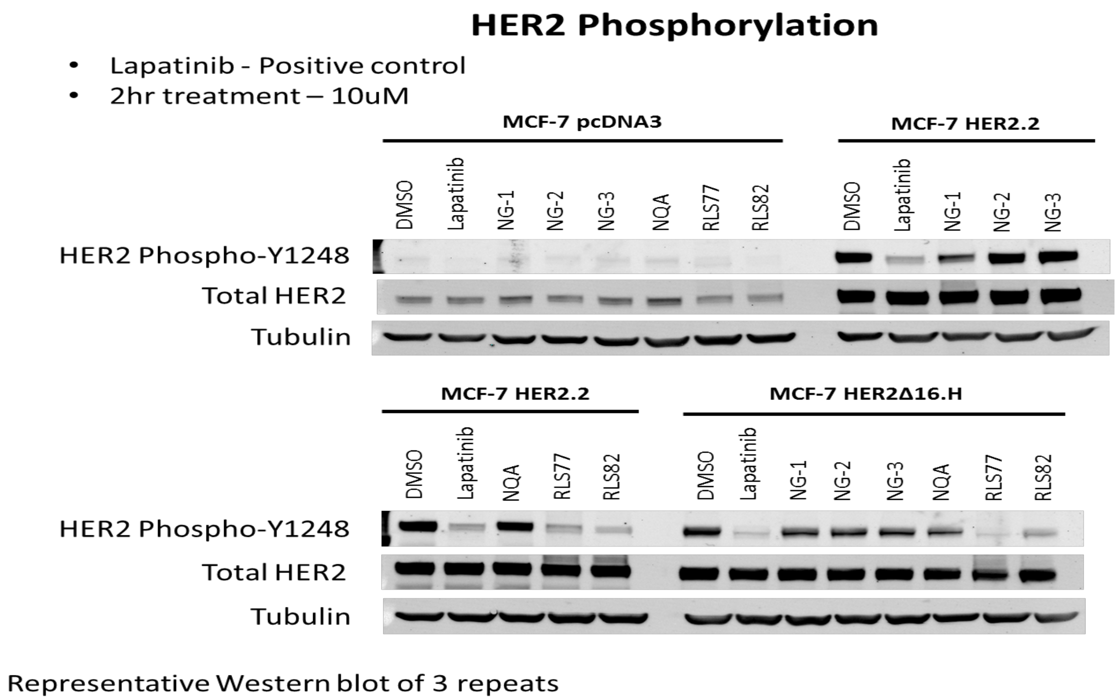


Figure 16: Western blot analysis of autophosphorylation at the HER2 residue Y1248. Compounds RLS77 and RLS82 inhibit the phosphorylation at Y1248 in MCF7/pcDNA, MCF/HER2 and MCF7/HER216.

To determine the ability of the drug treatments to inhibit the activation of receptor tyrosine kinase HER2, Western blots were performed to detect total phosphorylated

protein after treating each cell line with the three most potent drugs or lapatinib at 10μM for 2 hours (Figure 16). Similar to lapatinib, RLS77 and RLS82 were effective in suppressing the phosphorylation at Y1248 in each of the cell lines. NG-1, NG-2 and NG-3 did not show any such suppression indicating that that they did not inhibit HER2 protein.

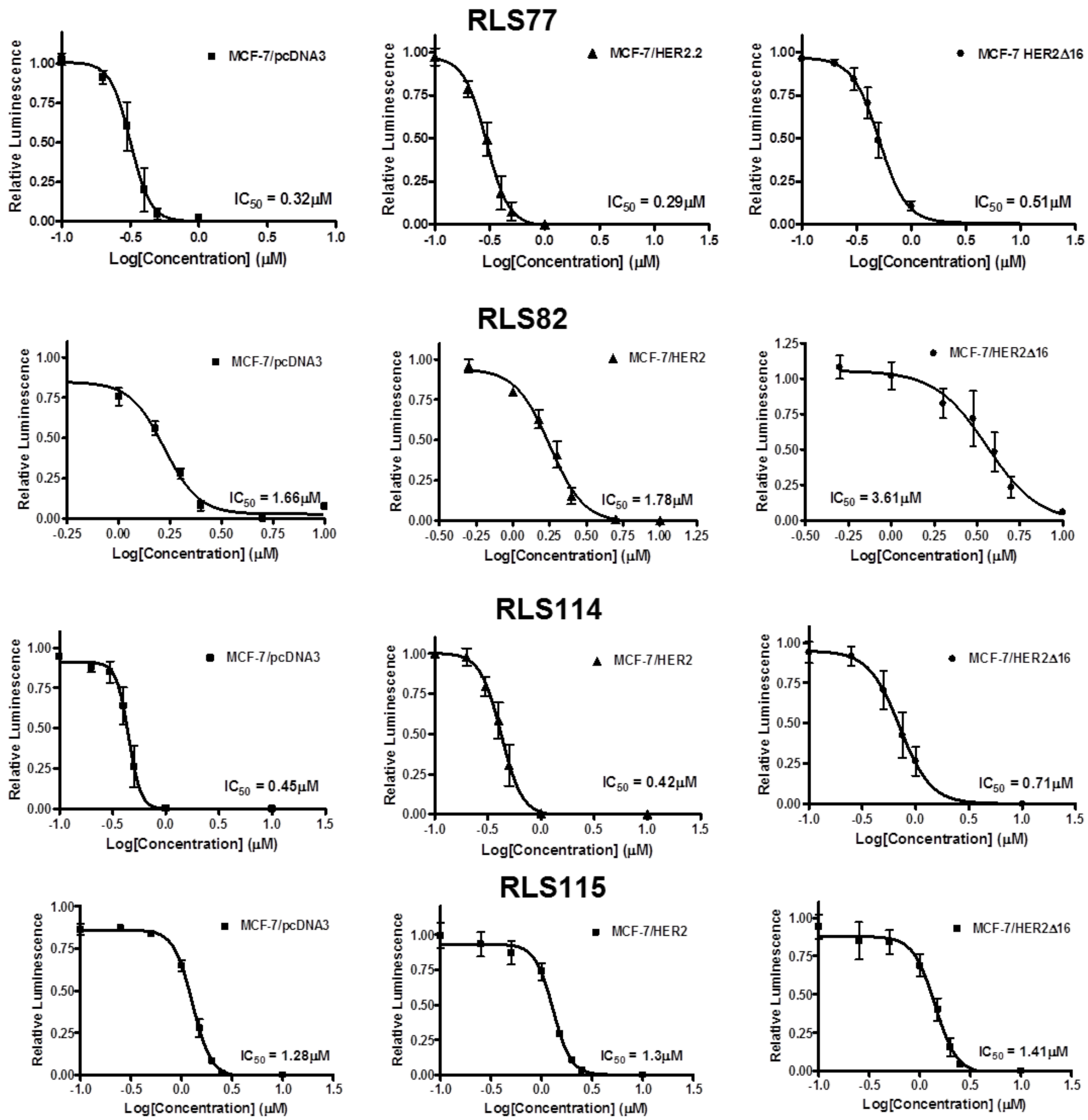


Figure 17: Inhibition of MCF-7/pcDNA, MCF-7/HER2 and MCF-7/HER2Δ16 cells by compounds RLS77, RLS82, RLS114 and RLS115.

The Dose-response curves were obtained for four of the compounds identified in the high-throughput screening against MCF7/pcDNA3, MCF7-HER2 and MCF7/HER2Δ16 (Figure 17). A graphical representation of the comparison of the IC50 values of growth

inhibition of MCF7/pcDNA3, MCF7-HER2 and MCF7/HER2Δ16 for these four compounds versus lapatinib is illustrated in figure 18. As can be seen, our compounds show much greater inhibition potency of these breast cancer cell lines than lapatinib.

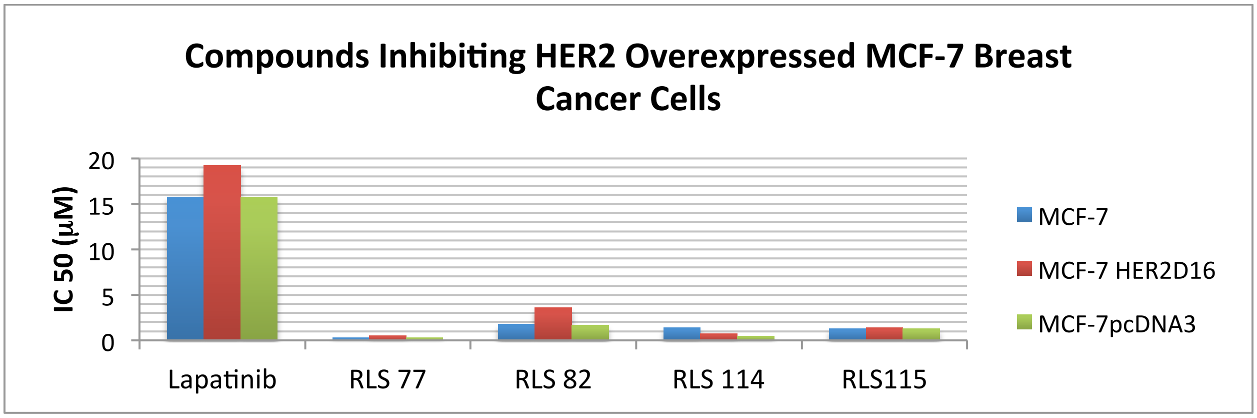


Figure 18: The IC50 values of RLS77, RLS82, RLS114 and RLS115 in comparison with that of lapatinib for growth inhibition of MCF7/pcDNA3, MCF7-HER2 and MCF7/HER2Δ16 cell lines.

Task 5- Determine preclinical activity and specificity of novel HER2-targeting molecules: determine influence of targeting molecules on HER2 oncogenic signaling and cellular responses using multiple validated preclinical models of breast tumorigenesis and metastasis. (Months 12-24)

Year 1: The compound NSC-322354 which showed the best inhibition activity against HER2Δ16 cell line was taken for an analysis of its cross kinase activity. The compound was sent to KinomeScan (www.kinomescan.com) for KINOMEscan's *in vitro* competition binding assay against a panel of 96 representative kinases. KINOMEscan™ is based on a competition binding assay that quantitatively measures the ability of a compound to compete with an immobilized, active site-directed ligand. The assay is performed by combining three components: DNA-tagged kinase; immobilized ligand; and a test compound. The ability of the test compound to compete with the immobilized ligand is measured via quantitative PCR of the DNA tag (description of method taken from www.kinomescan.com). The compound was tested at a concentration of 10 mM. The results are given in Table 3. The S-score, selectivity score (the number of kinases that bind to the compound divided by the total number of distinct kinases tested), which is a quantitative measure of the compound selectivity, was 0.022. The compound showed good selectivity for two of the kinases, Casein Kinase 1 D and PIM kinases (more selective for PIM1 and PIM3 kinases). Both of these kinases are serine/threonine kinases. PIM1 is an oncogene. The PIM1 gene was initially identified as a proviral integration site in Moloney Murine leukemia virus-induced mouse T-cell lymphomas[2, 3]. Pim kinases are implicated in the development of solid tumors. DNA microarray

analyses showed the overexpression of PIM1 in human prostate cancer in relation to the grade of the prostate cancer. CK1d is a member of the ubiquitous casein kinase-1 family, and alterations in the expression and/or activity of CK1 have been observed in breast cancer[4]. CK1d, has been identified as a novel kinase implicated in the modulation of physiological aspects of both ERa (estrogen receptor alpha) and AIB1 (amplified in breast cancer-1 protein). The compound in fact did not show good inhibition of HER2 (ERBB2) kinase. The *in vitro* high-throughput assay of the compounds is currently being performed for the proteins PIM1 kinase, and casein kinase 1 D. This will be followed by a dose response curve assay to determine the IC_{50} value for these kinases. These compounds will also be tested for inhibition of other breast cancer cell lines.

Table 3. Matrix of Compound NSC322354 Screen

Kinase target	%Control @ 10μM		Kinase target	%Control @ 10μM
ABL1(E255K)-phosphorylated	96		KIT(V559D,T670I)	86
ABL1(T315I)-phosphorylated	95		LKB1	100
ABL1-phosphorylated	88		MAP3K4	100
ACVR1B	90		MAPKAPK2	93
ADCK3	100		MARK3	100
AKT1	100		MEK1	92
AKT2	100		MEK2	96
ALK	100		MET	100
AURKA	87		MKNK1	45
AURKB	72		MKNK2	98
AXL	100		MLK1	99
BMPR2	60		p38-alpha	100
BRAF	87		p38-beta	92
BRAF(V600E)	70		PAK1	93
BTK	100		PAK2	57
CDK11	86		PAK4	91
CDK2	92		PCTK1	100
CDK3	88		PDGFRA	100
CDK7	88		PDGFRB	100
CDK9	100		PDPK1	87
CHEK1	79		PIK3C2B	100
CSF1R	77		PIK3CA	99
CSNK1D	30		PIK3CG	68
CSNK1G2	100		PIM1	34
DCAMKL1	90		PIM2	67
DYRK1B	53		PIM3	36
EGFR	88		PKAC-alpha	84
EGFR(L858R)	78		PLK1	96
EPHA2	100		PLK3	54
ERBB2	93		PLK4	89
ERBB4	95		PRKCE	92
ERK1	100		RAF1	100
FAK	95		RET	100
FGFR2	100		RIOK2	86
FGFR3	93		ROCK2	77
FLT3	84		RSK2(Kin.Dom.1-N-terminal)	53
GSK3B	85		SNARK	90
IGF1R	100		SRC	91
IKK-alpha	87		SRPK3	93
IKK-beta	91		TGFBR1	100
INSR	97		TIE2	89
JAK2(JH1domain-catalytic)	94		TRKA	78
JAK3(JH1domain-catalytic)	61		TSSK1B	89
JNK1	96		TYK2(JH1domain-catalytic)	57
JNK2	84		ULK2	88
JNK3	76		VEGFR2	100
KIT	76		YANK3	89
KIT(D816V)	100		ZAP70	100

Year 2: A total of 40 compounds were subjected to high-throughput screening for inhibition of Pim1 kinase though an *in vitro* kinase assay. Out of these 9 compounds (Figure 14) showed notable inhibition potency ranging from 1.3 μM to 57.1 μM (Table 4). Four of these compounds were shown to inhibit the prostate cancer cell line DU-145 cells with inhibition potencies similar to that of emodin (Figure 15).

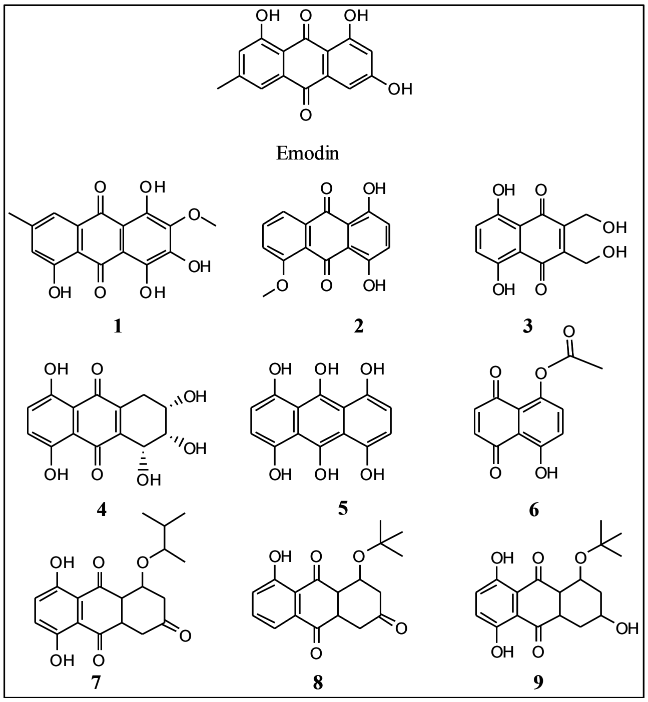


Figure 19: Structure of emodin and compounds 1 to 9 investigated for inhibition of Pim 1 kinase.

Compound	IC_{50} (μM)
Emodin	2.5
1	21.4
2	11.6
3	7.4
4	19.2
5	57.1
6	1.3
7	3.6
8	23.8
9	3.5

Table 4: Inhibition of Pim1 kinase by emodin and compounds **1** to **9**.

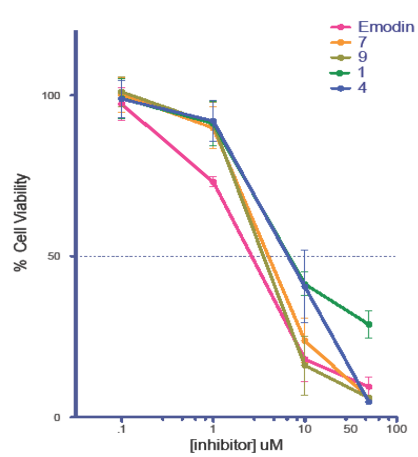


Figure 20: Growth inhibition curve for DU-145 cells treated with compounds 1, 4, 7, 8 and Emodin.

Year 3: Toxicology assay on DU-145 cells for the compounds in Table 4

DU-145 cells were seeded in a 96-well plate at 0.9x105 cell/ml. The next day the media is replaced with 0.2% FBS media for 72 hours. At this point, an Alamar Blue assay is performed to calculate cell number per well. Drug is then added and the cells are counted again after an additional 72 hours. The ratio between the number of cells before drug and after incubation with drug is then calculated. Emodin, compounds **7** and **9** were found to exhibit toxicity on these cells (Figure 16).

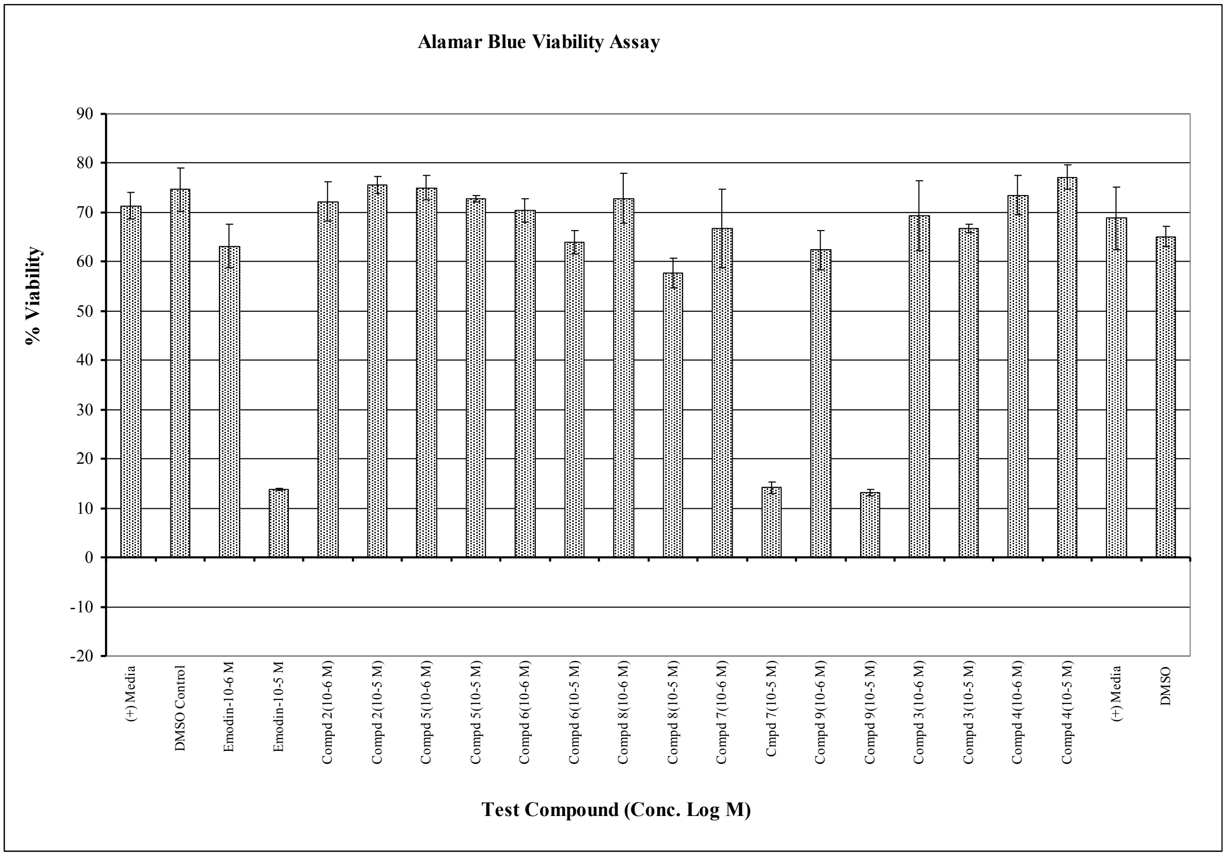


Figure 21: Toxicology assay on DU-145 cells for emodin and the compounds **1-9** in Table 4.

Year 4: The compound RLS77 was chosen for further studies due to its high potency of inhibition of the MCF7/HER2 and MCF7-HER2D16 cell lines (Figure 18). This compound was tested against many other breast cancer cell lines that were trastuzumab sensitive (SKBR3 and BT474), trastuzumab resistant (SUM190PT and SUM225CWN) and triple negative (BT20, MDA-MB-468 and MDA-MB-231). The growth inhibition potency for all of these cell lines was similar to the MCF7 cell line (0.12 to 0.75 μM) except for MDA-MB-231 with an IC50 value of 2.92 μM (Figure 22).

	Cell Line	RLS77 IC_{50} (uM)
	MCF-7	0.32
Triple Negative	BT20	0.4626
	MDA-MB-468	0.7447
	MDA-MB-231	2.923
Trastuzumab Sensitive	SKBR3	0.4274
	BT474	0.3062
Trastuzumab Resistant	SUM 190PT	0.6321
	SUM 225CWN	0.1231

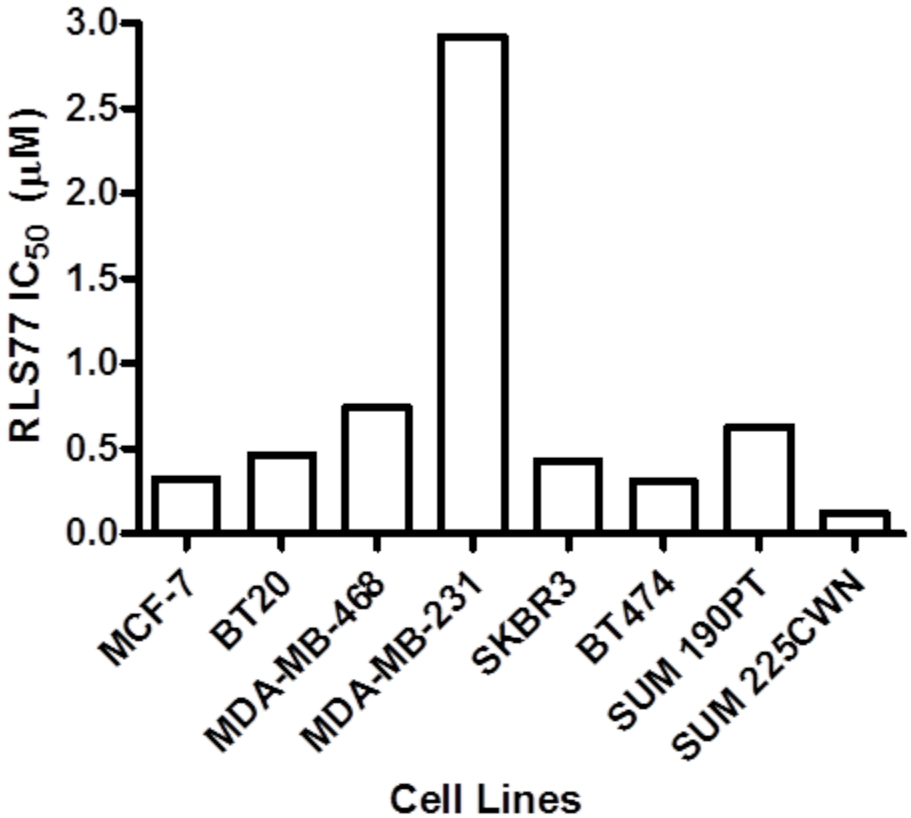


Figure 22: Growth inhibition of various breast cancer cell lines by the compound RLS77.

5a - Perform genome-wide shRNA library screen coupled with gene expression arrays of sensitive cells to identify Drug Targets, Drug Sensitizers, and Drug Resistance Pathways. (Months 18-30)

Not initiated.

5b - Identify and validate drug combinations to improve efficacy and overcome resistance in preclinical models. (Months 24-36)

Not initiated.

5c - Confirm efficacy of drug combinations in preclinical *in vivo* xenograft and transgenic mouse models of breast cancer. (Months 30-48)

The IACUC application has been approved. The experiments will be initiated shortly. We will start the *in vivo* xenograft experiments with the BT474 and SKBR3 cell lines.

<u>Programmatic Activities</u>

The breast cancer research group members have met multiple times to discuss and plan the various aspects of the Project. A number of seminars/workshops were also held for the group:

1- Monday September 8, 2014, Cecily DeFreece, Ph.D., Department of Biology, Xavier University of Louisiana
2- Monday September 15, 2014, Thomas Huckaba, Ph.D., Department of Biology, Xavier University of Louisiana
3- Monday September 22, 2014, Guangdi Wang, Ph.D., Department of Chemistry, Xavier University of Louisiana
4- Monday September 29, 2014, Guangdi Wang, Ph.D., Department of Chemistry, Xavier University of Louisiana
5- Monday October 6, 2014, Lee Roy Morgan, Ph.D., DEKK-TEC, Inc.
6- Monday October 20, 2014, Akash Gupta, Ph.D., Northwestern University
7- Monday October 27, 2014, Syreeta Tilghman, Ph.D., College of Pharmacy, Xavier University of Louisiana
8- Monday November 3, 2014, Zhe Wang, Ph.D., Department of Chemistry, Xavier University of Louisiana
9- Monday November 10, 2014, Zongbing You, M.D., Ph.D., Tulane University
10- Monday November 17, 2014, Jaya Sridhar, Ph.D., Department of Chemistry, Xavier University of Louisiana
11- Monday November 24, 2014, Partha Chandra, Ph.D., Tulane University
12- Monday December 1, 2014, Robert Stratford, Ph.D., College of Pharmacy, Xavier University of Louisiana

13- Monday December 8, 2014, Florastina Payton-Stewart, Ph.D. and Ravi Pingali, Ph.D., Department of Chemistry, Xavier University of Louisiana
14- Monday December 15, 2014, Anup Kundu, Ph.D., Department of Biology, Xavier University of Louisiana
15- Monday February 2, 2015, Guandgi Wang, Ph.D., and Thomas Wiese, Ph.D., Department of Chemistry and College of Pharmacy, Xavier University of Louisiana
16- Monday March 2, 2015, Melyssa Bratton, Ph.D., LCRC Cell and Molecular Core, Xavier University of Louisiana
17- Monday March 9, 2015, Matthew Burow, Ph.D., Tulane University
18- Monday March 16, 2015, Cecily DeFreece, Ph.D., Department of Biology, Xavier University of Louisiana
19- Monday March 23, 2015, Kristine J. Kines, Ph.D., Tulane University
20- Monday April 6, 2015, Kelly Johanson, Ph.D., Department of Chemistry, Xavier University of Louisiana
21- Monday April 13, 2015, Florastina Payton-Stewart, Ph.D., Department of Chemistry, Xavier University of Louisiana
22- Monday April 20, 2015, Michael Johnson, Ph.D., Director of the Louisiana Campaign for Tobacco-Free Living
23- Monday April 27, 2015, Kun Zhang, Ph.D., Department of Computer Science, Xavier University of Louisiana

In addition, the researchers on each subproject (faculty, staff, and students) have met multiple times during the past grant year to discuss project direction and experimental designs and results for their projects.

KEY RESEARCH ACCOMPLISHMENTS:

<u>Foroozesh/Beckman/Burow Subproject</u>

- Successfully developed a facile synthetic route to prepared 3-ketone-4,6-diene and 1-position-modified ceramide analogs, and obtained 16 novel ceramide analogs.
- Discovered a highly potent ceramide analog (406). The mechanism investigation showed that analog 406 leads to cell apoptosis through intrinsic apoptotic pathway and does not interrupt the function of GCS.
- Designed, synthesized, and determined a novel GCS inhibitor (503, a 1-position-modified ceramide analog), which is extremely useful for the development of highly potent GCS inhibitors.
- Successfully developed a facile synthetic route to prepare fluorescent building blocks for ceramide analogs. Eleven pyranoflavones, three furanoflavones, one pyridinoflavone, one dioxoloflavone, four pyranocoumarin, four furanocoumarin, one pyridinocoumarin, and one dioxolocoumarin were synthesized.

- Discovered the conformational isomerism of oxazolidine ceramide analogs. According to the evidences from NMR spectra, successfully constructed the molecular models of two conformational isomers.
- Discovered highly potent and selective cytochrome P450 1A1 inhibitors, 4-ethynylflavone and 6-ethynylflavone.
- Discovered selective P450 1A2 inhibitors, 7,8-furanoflavone and 7,8-pyrano-4-trifluoromethylcoumarin.

Wiese/Burow Subproject

- Identified representative ER LBD structures to be used for virtual screening.
- Identified optimal ligand receptor (Docking) method for virtual screening.
- Obtained phytochemical and marketed drug databases for processing.
- Trained three pharmacy students, one in molecular modeling, and both in bioassays.
- Developed new, comprehensive method for virtual screening with ER LBDs.
- Trained one foreign exchange pharmacy student in bioassays.
- Obtained *in vitro* ER agonist and antagonist activity data for 29 naturally occurring stilbenes.
- 5 stilbenes have been characterized as ER agonists, 3 as ER antagonists.
- Modeling of potential binding modes suggest that antagonist stilbenes may act as direct and indirect antagonists of ER.

Sridhar/Jones/Stevens Subproject

- Synthesized several new naphthoquinone derivatives as potential growth inhibitors of MCF7-HER2 and MCF7-HER2Δ16 breast cancer cell lines.
- Performed western blot analysis of autophosphorylation at the HER2 residue Y1248. Identified two compounds RLS77 and RLS82 that inhibit the phosphorylation at Y1248 in MCF7/pcDNA, MCF/HER2 and MCF7/HER2Δ16.
- Determined IC50 values of growth Inhibition of MCF-7/pcDNA, MCF-7/HER2 and MCF-7/HER2Δ16 cells by compounds RLS77, RLS82, RLS114 and RLS115.
- Performed further studies to determine the growth inhibition efficiency of the compound RLS77 against many other breast cancer cell lines that were trastuzumab sensitive (SKBR3 and BT474), trastuzumab resistant (SUM190PT and SUM225CWN) and triple negative (BT20, MDA-MB-468 and MDA-MB-231).
- Obtained approval of IACUC application to initiate *in-vivo* xenograft experiments with the breast cancer cell lines.
- Identified three compounds that inhibit MCF7-pcDNA, MCF7-HER2Δ16 and MCF7-HER2 overexpressing breast cancer cell lines with sub-micromolar potency.

- Performed Western blots to detect total phosphorylated protein after treating each cell line with the three most potent drugs. IC_{50} values for the three compounds were measured for MCF7-pcDNA, MCF7-HER2Δ16 and MCF7-HER2 cell lines.
- Performed docking studies on the identified compounds revealing the two hydrogen bonds made by the quinones with the HER2 kinase. The first hydrogen bond made by all three compounds was to the hinge region residue Gln799. The second hydrogen bond was formed by the two compounds showing higher potency to one of the two residues- invariable Lys753 or Asp863.
- Discovered two new lead compounds for derivatization LM1 and LM2.
- Established synthetic protocols for the synthesis of different derivatives of LM1. The synthetic scheme for LM2 synthesis is presently being standardized.
- Performed Western blot analysis of autophosphorylation at the EGFR residue Y1068 and HER2 residue Y1248.
- Determined IC50 values of inhibition for HER2 and EGFR kinases.
- Performed Toxicology assays on HeLa cells and DU-145 cells
- Determined the lead structure for achieving inhibition of MCF7-HER2Δ16 and MCF7-HER2 overexpressing breast cancer cell lines while limiting toxicity.
- Found a new core structure 1H-indazol-3-ol/indazolone as a potential inhibitor through computational database searches.
- Synthesized several quinone derivatives and three indazolone derivatives that will be subjected to in-vitro inhibition assays against MCF7 cell line.

<u>Program Accomplishments</u>

- Organized meetings in order to introduce the students, staff, and faculty members working on the different subprojects to each other and the various projects.
- Organized meetings for each subproject’s researchers to meet to discuss project direction and experimental designs and results.
- Organized training workshops/seminars.

REPORTABLE OUTCOMES:

- **Publications**

<u>Foroozesh/Beckman/Burow Subproject</u>

“Cytochrome P450 Family 1 Inhibitors and Structure-Activity Relationships”, J. Liu, J. Sridhar, and M. Foroozesh, *Molecules,* ***18*** **(12)**, 14470-14495, 2013.

“A Review of Ceramide Analogs as Potential Anticancer Agents”, J. Liu, B.S. Beckman, and M. Foroozesh, *Future Med Chem*, **5**, 1405-1421, 2013.

“Pyranoflavones: a group of small-molecule probes for exploring the active site cavities of cytochrome P450 enzymes 1A1, 1A2, and 1B1”, J Liu, S. Taylor, P. Dupart, C. Arnold, J. Sridhar, Q. Jiang, Y. Wang, E. Skripnikova, M. Zhao, and M. Foroozesh, *J Med Chem*, **56**, 4082-4092, 2013.

“3-Ketone-4,6-Diene Ceramide Analogs Exclusively Induce Apoptosis in Chemo-Resistant Cancer Cells”, A. Ponnapakkam, J. Liu, K. Bhinge, B. Drew, T. Wang, J. Antoon, T. Nguyen, P. Dupart, Y. Wang, M. Zhao, Y.Y. Liu, M. Foroozesh, and B. Beckman, *Bioorg Med Chem*, ***22*** **(4)**, 1412-1420, 2014.

“Ethynylflavones, Highly Potent and Selective Inhibitors of Cytochrome P450 1A1”, N. Goya, J. Liu, L. Lovings, P. Dupart, S. Taylor, S. Bellow, L. Mensah, E. McClain, B. Dotson, J. Sridhar, X. Zhang, M. Zhao, and M. Foroozesh, *Chem Res Toxicol,* **27(8)**, pp. 1431–1439, 2014.

“A Ligand-Based Drug Design. Discovery of 4-Trifluoromethyl-7,8-pyranocoumarin as a Selective Inhibitor of Human Cytochrome P450 1A2”, J. Liu, P.T. Pham, E.V. Skripnikova, S. Zheng, L.J. Lovings, Y. Wang, N. Goyal, S.M. Bellow, L.M. Mensah, A.J. Chatters, M.R. Bratton, T.E. Wiese, M. Zhao, G. Wang, M. Foroozesh, *J Med Chem*, E-published, DOI: 10.1021/acs.jmedchem.5b00494, 2015.

<u>Wiese/Burow Subproject</u>

None yet.

<u>Sridhar/Jones/Stevens Subproject</u>

“Identification of Quinones as HER2Δ16 and HER2 inhibitors for the Treatment of Trastuzumab Breast Cancer” Jayalakshmi Sridhar, Mary Sfondouris, Thuy-Linh Nguyen, Ian Townley, Cheryl Stevens, Frank Jones. Bioorg. Med. Chem. Lett., 2014 Jan 1; 24(1):126-31. doi: 10.1016/j.bmcl.2013.11.064. Epub 2013 Dec 3.

“Small Molecule Tyrosine Kinase Inhibitors of ErbB2/HER2/Neu in Aggressive Metastatic Breast Cancer” Richard L. Schroeder, Cheryl K. Stevens and Jayalakshmi Sridhar. *Molecules*, **2014** Sep 23; 19(9):15196-15212.

“*Ortho*-Methylarylamines as Potential Mechanism-Based Inhibitors of Cytochrome P450 1A1 and 1A2 Enzymes”. Jayalakshmi Sridhar*, Jiawang Liu, Quan Jiang, Richard Schroeder, Nancy Pham, Phan Tram, Kevin Riley, and Maryam Foroozesh. Manuscript submitted to *Journal of Applied Toxicology*.

“Novel functionalized 5-(phenoxymethyl)-1,3-dioxane analogs exhibiting cytochrome P450 inhibition: a patent evaluation WO2015048311 (A1)”. Richard L. Schroeder, Phan Tram, Jiawang Liu, Maryam Foroozesh, Jayalakshmi Sridhar*. Manuscript submitted to *Expert Opinion in Therapeutic Patents,* under review.

“Ethynylflavones, Highly Potent and Selective Inhibitors of Cytochrome P450 1A1. Goyal, N; Liu, J; Lovings, L; Dupart, P; Taylor, S; Bellow, S; Mensah, L; McClain, E; Dotson, B; Sridhar, J; Zhang, X; Zhao, M; Foroozesh, M*”. *Chem. Res. Toxicol.,* **2014** Jul 29.

“Quinones as New Dual Inhibitors of Pim 1 Kinase and Casein Kinase 2”. Richard L. Schroeder, Navneet Goyal, Melyssa Bratton, Ian Townley, Nancy Pham, Phan Tram, Treasure Stone, Kathy Nguyen, and Jayalakshmi Sridhar*. Manuscript under preparation.

“Functionalization and Modification of Naphthoquinone Analogs as HER2 Kinase Inhibitors” Divya Jyothi Lella, M.S. Thesis, Western Kentucky University, May 2014.

- **Presentations**

Foroozesh/Beckman/Burow Subproject

“The Design and Synthesis of Benzoate Esters as Potential Anti-proliferation Agents and Inhibitors of Cytochrome P450 Enzymes”, C. Arnould, P. Dupart, J. Liu, and M. Foroozesh, the Annual LaSPACE Council Meeting, and the American Chemical Society Local Section Student Poster Presentation, New Orleans, LA, October 2012.

“Propargyl Flavones as Inhibitors of Human Cytochrome P450s 1A1, and 1A2”, S. Taylor, J. Liu, P. Dupart, and M. Foroozesh, the American Chemical Society Local Section Student Poster Presentation, New Orleans, LA, October 2012.

“Quest for New Mechanism-Based Inhibitors of Cytochrome P450 Enzymes 1A1 and 1A2”, J. Sridhar, J. Liu, M. Foroozesh, C.L. Stevens, the Louisiana Cancer Research Consortium Annual Retreat, New Orleans, LA, March 2013.

“Pyranoflavones and 5-Hydroxy-pyranoflavones as Small-molecule Probes into the Active Site Cavities of P450s 1A1 and 1A2”, J. Liu, S. Taylor, P. Dupart, C. Arnold, and M. Foroozesh, the Louisiana Cancer Research Consortium Annual Retreat, New Orleans, LA, March 2013.

“Design, Synthesis and Evaluation of 3-Oxy-substituted Pyridine Terminal Alkynes”, Q. Jiang, J. Sridhar, M. Minaruzzaman, J. Liu, and M. Foroozesh, the Louisiana Cancer

Research Consortium Annual Retreat, New Orleans, LA, March 2013.

"Design, Synthesis, and Bioassays of Potential P450 Inhibitors", M. Foroozesh, Invited Oral Presentation at the American Chemical Society National Meeting, New Orleans, LA, April 2013.

"Quest for New Mechanism-Based Inhibitors of Cytochrome P450 Enzymes 1A1 and 1A2", J. Sridhar, J. Liu, M. Foroozesh, C.L. Stevens, the American Chemical Society National Meeting, New Orleans, LA, April 2013.

"The Design and Synthesis of Benzoate Esters as Potential Anti-proliferation Agents and Inhibitors of Cytochrome P450 Enzymes", C. Tutwiler, C. Arnold, P. Dupart, J. Liu, and M. Foroozesh, the American Chemical Society National Meeting, New Orleans, LA, April 2013.

"Ethynyl Flavones as Inhibitors of Cytochrome P450 Enzymes", S. Taylor, J. Liu, P. Dupart, and M. Foroozesh, the American Chemical Society National Meeting, New Orleans, LA, April 2013.

"Flavone Derivatives as Small-molecule Probes of Cytochrome P450 Enzymes: Inhibitory Activity and Selectivity", J. Liu, S. Taylor, P. Dupart, C. Arnold, and M. Foroozesh, the American Association for Cancer Research Annual Meeting, Washington, D.C., April 2013.

"Ceramide Analogs as Resistant Cancer Cell Killing Agents, and Their Ability to Inhibit Glucosylceramide Synthase", J. Liu, A. Ponnapakkam, K. Bhinge, Y. Liu, B. Beckman, M. Foroozesh, the Louisiana Cancer Research Consortium Annual Retreat, New Orleans, LA, March 2014.

"Synthesis and biological studies of a new series of ethynylflavones as cytochrome P450 inhibitors", N. Goyal, J. Liu, M. Foroozesh, the Louisiana Cancer Research Consortium Annual Retreat, New Orleans, LA, March 2014.

"The Design and Synthesis of Resorufin Propargyl Ethers as Potential Cytochrome P450 Inhibitors", S. Bellow, L. Lovings, J. Liu, and M. Foroozesh, the American Chemical Society National Meeting, Dallas, TX, April 2014.

"The Design and Synthesis of Benzoate Esters as Potential Anti-proliferation Agents and Inhibitors of Cytochrome P450 Enzyme", E. McClain, L. Lovings, J. Liu, and M. Foroozesh, the American Chemical Society National Meeting, Dallas, TX, April 2014.

"Synthesis of Resorufin Derivatives as Inhibitor Indicators of Cytochrome P450 Enzymes", L. Lovings, J. Liu, and M. Foroozesh, the American Chemical Society National Meeting, Dallas, TX, April 2014.

"Pyrano- and Furanochromones as Specific Inhibitors of Human Cytochrome P450 1A2" J. Liu, P. Pham, L. Lovings, M. Foroozesh, ACS 249th National Meeting & Exposition, Denver, CO, Mar 21-25, 2015.

<u>Wiese/Burow Subproject</u>

"*In vitro* estrogen activity of 29 stilbenes: Structure-activity relationships for agonist and antagonist activity", T. Mitchell, G. Barbarini, P. Ma, M. Burow, and T. Wiese, the American Chemical Society National Meeting, Dallas, TX, April 2014.

<u>Sridhar/Jones/Stevens Subproject</u>

"Design and Development of Selective Pim1 Kinase Inhibitors", Jasmine Thompson, Ian Townley, C.L. Stevens and J. Sridhar. ACS National Meeting, New Orleans, April 2013.

"Functionalization and modification of Shikonin compounds as HER2 inhibitors", D.J. Lella, J. Sridhar, C.L. Stevens, and B. Yan. Western Kentucky University – Reach Week poster, April 2013.

"Functionalization and modification of 2-hydroxymethyl-5, 8-dimethoxy-1, 4-naphthaquinone as HER2 inhibitors", D.J. Lella, J. Sridhar, C.L. Stevens, and B. Yan. ACS National Meeting, Indianapolis, Sept 2013.

"New Quinones as HER2 inhibitors for the Treatment of Trastuzumab Resistant Breast Cancer". Schroeder R, Sfondouris M. Jones F E, Bratton M, Townley I, Stevens C L K, Nguyen T-L K*, Nguyen D Q*, Simmons D*, Thompson J* and Sridhar J; ACS National Meeting, Dallas, TX, March 2014.

"Synthesis of 1 H-indazol-3(2H)-one Derivatives as Potential CDK Inhibitors". Pham N*, Schroeder R, Tram P*, Nguyen T-L K*, Jennings S*, Vu T*, McFerrin H E and Sridhar J; ACS National Meeting, Dallas, TX, March 2014.

"Synthesis of 1 H-indazol-3(2H)-one Derivatives as Potential CDK Inhibitors". Pham N*, Schroeder R, Tram P*, Nguyen T-L K*, Jennings S*, Vu T*, McFerrin H E and Sridhar J; LCRC Retreat, New Orleans, LA, March 2014.

"Drug Resistant HER216 overexpression cells are sensitive to a new class of tyrosine kinase inhibitors", Mary E. Sfondouris, Jayalakshmi Sridhar, Melyssa R Bratton, Cheryl L. Klein Stevens, Frank E. Jones. LCRC Annual Scientific Retreat 2014, New Orleans, Louisiana, March 2014.

“Development of Casein Kinase 1 Inhibitors”. Schroeder R, Bratton R, Skripnikova E, Thompson J*, Nguyen D Q*, Nguyen K*, Sridhar J; LCRC Retreat, New Orleans, LA, March 2014.

“Synthesis of 1 H-indazol-3(2H)-one Derivatives as Potential CDK Inhibitors.” Pham N*, Tram P*, Geathers J*, Nguyen T-L K*, Schroeder R, Jennings S*, Vu T*, McFerrin H E, Sridhar J; Festival of Scholars at Xavier University of Louisiana, New Orleans, Louisiana, April 2014.

“Synthesis and Characterization of Phthalimide Derivatives as CDK Inhibitors”. Tram P*, Geathers J*, Schroeder R, Sridhar J; Festival of Scholars at Xavier University of Louisiana, New Orleans, Louisiana, April 2014.

“Development of Quinones as Casein Kinase Inhibitors”. Nguyen K H*, Nguyen D Q*, Schroeder R, Sridhar J; Festival of Scholars at Xavier University of Louisiana, New Orleans, Louisiana, April 2014.

“Computational investigation of alpha selective LXR ligands”. Theard B*, Gibson T*, Ndukwe M*, Riley K, and Sridhar J; LaSigma Poster Presentation, Tulane University, New Orleans, Louisiana, July 2014.

“Specific Ligand-Residue Interactions that Lead to Liver X Receptor Isoform Selectivity”. Kathryn M. Hardin, Susannah L. Davis, Kevin E. Riley, Jayalakshmi Sridhar. 2015 LA-SiGMA Symposium, Baton Rouge, LA, July 2015.

- **Employment or Research Opportunities**

Individuals trained in the first and second years of this DoD breast cancer project:

Jiawang Liu, Postdoctoral Fellow at Xavier University (Foroozesh Lab)

Jayalakshmi Sridhar, Postdoctoral Fellow at Xavier University (Stevens Lab, currently a new tenure-track faculty member at the Xavier University Department of Chemistry)

James Antoon, Medical Student at Tulane University (Beckman Lab, received his M.D. in May 2012 after receiving his Ph.D. in 2010 at Tulane University. He is currently doing his residency in pediatrics at the University of North Carolina.)

Barbara Drew, Medical Student at Tulane University (Beckman Lab, is currently in her residency in obstetrics and gynecology in Connecticut.)

Tony Wang, Medical Student at Tulane University (Beckman Lab, working on this DoD subproject)

Thong T. Nguyen, Undergraduate Student at Xavier University (Foroozesh Lab, graduated in May 2012 and is now pursuing a Ph.D. in Chemistry at Tulane University)

Adharsh P. Ponnapakkam, Undergraduate Student at Tulane University (Beckman Lab, graduated in May 2012 and is currently continuing his work on this DoD project as a Masters student at Tulane University)

Patrick Dupart, Technician at Xavier University (Foroozesh Lab, Xavier graduate, joined Virginia Commonwealth University as a graduate student in July of 2013)

Shannon Taylor, Technician at Xavier University (Foroozesh Lab, Xavier graduate)

Corey Arnold, Undergraduate Student at Xavier University (Foroozesh Lab)

Erika McClain, Undergraduate Students at Xavier University (Foroozesh Lab)

Brandon Dotson, Undergraduate Students at Xavier University (Foroozesh Lab)

Charne’sa Tutwiler, Undergraduate Students at Xavier University (Foroozesh Lab)

La’Nese Lovings, Technician at Xavier University (Foroozesh Lab, Xavier graduate)

Megan McKay, Undergraduate Students at Xavier University (Foroozesh Lab)

Sydni Bellow, Undergraduate Students at Xavier University (Foroozesh Lab)

Lydia Mensah, Undergraduate Students at Xavier University (Foroozesh Lab)

Amari Chatters, Undergraduate Students at Xavier University (Foroozesh Lab)

Peter Pham, Undergraduate Students at Xavier University (Foroozesh Lab)

Candace Hopgood, Technician at Xavier University (Wiese Lab, Xavier graduate, started Xavier Pharmacy School in August 2013)

Peng Ma, Technician at Xavier University (Wiese Lab)

Chioma Obih, Pharmacy Student at Xavier University (Wiese Lab, graduated in May 2013)

Gabriela Barbarini, Pharmacy Exchange Student at Xavier University (Wiese and Burow Labs)

Felicia Gibson, Pharmacy Student at Xavier University (Wiese Lab)

Tamara Mitchell, Pharmacy Student at Xavier University (Wiese Lab)

Gerald Guirard, Pharmacy Student at Xavier University (Wiese Lab)

Elizabeth Martin, Graduate Student at Tulane University (Burow Lab)

Felicia Huynh, Graduate Student at Tulane University (Jones Lab)

Hope Burks, Graduate Student at Tulane University (Burow Lab)

Lucas Chan, Masters Student at Tulane University (Beckman Lab)

Lyndsay Rhodes, Postdoctoral Fellow at Tulane University (Burow Lab)

Melyssa Bratton, Instructor at Tulane University (Burow Lab, currently Research Associate at Xavier University Pharmacy)

Steven Elliott, Lab Supervisor at Tulane University (Burow Lab)

Van Hoang, Graduate Student at Tulane University (Burow Lab)

Han Wen, Graduate Student at Tulane University (Jones Lab)

Mary Sfondouris, Postdoctoral Fellow at Tulane University (Jones Lab)

Thuy-Linh Nguyen, Undergraduate Student at Xavier University (Sridhar lab)

Jasmine Thompson, Undergraduate Student at Xavier University (Sridhar lab)

Lella Divya Jyothi, Graduate Student at Western Kentucky University (Stevens lab)

Marleesa Bastian, Technician at Xavier University (Sridhar lab and is now pursuing graduation at Meharry Medical College school of Medicine, Tennessee)

Richard Schroeder, Research Technician at Xavier University (Sridhar lab)

Nancy Pham, Undergraduate Student at Xavier University (Sridhar lab)

Phan Tram, Undergraduate Student at Xavier University (Sridhar lab)

Jasmine Geathers, Undergraduate Student at Xavier University (Sridhar lab)

Don Q. Nguyen, Undergraduate Student at Xavier University (Sridhar lab)

Treasure Store, Research Technician at Xavier University (Sridhar lab)

Faith Joseph, Research Technician at Xavier University (Sridhar lab)

Mariya Iqbal, Research Technician at Xavier University (Sridhar lab)

Veronica Miles, Undergraduate Student at Xavier University (Sridhar lab)

Kathy Nguyen, Undergraduate Student at Xavier University (Sridhar lab)

Kathryn M. Hardin, REU Summer Research Student at Xavier University (Sridhar lab)

CONCLUSION:

Foroozesh/Beckman/Burow Subproject

In Year 1, our results have shown that extending the conjugated system in the backbone of ceramide analogs can lead to an increase in the anti-cancer activity. This observation is expected to assist us in designing more potent anti-cancer ceramide analogs.

We have also discovered the conformational isomers of pro-apoptotic ceramide analogs, 401 and 402. This isomerism leads to the possibility that oxazolidine ceramide analogs could act on their molecular targets with two conformations. This finding provides us with a new perspective for the investigation of ceramide-receptor interactions.

In Year 2, we found that the modification of the 1-position of ceramides can lead to novel glucosylceramide synthase (GCS) inhibitors. This finding provides us with a new perspective for the design of effective GCS inhibitors. However, inhibition of GCS is not directly correlated with the ability of ceramide analogs to selectively kill chemo-resistant cancer cell. This indicates that ceramide analogs could inhibit cancer multi-drug resistance through two pathways.

In Years 2 and 3, we synthesized **26** novel fluorescent flavone and coumarin derivatives, which are important building blocks of fluorescently visible ceramide analogs. These various fluorescent building blocks include pyranoflavones, furanoflavones, dioxolofavones, pyridinoflavones, pyranocoumarins, furanocoumarins, dioxolocoumarins, and pyridinocoumarins.

In Year 4, we tested **32** flavone and coumarin derivatives on the inhibition of P450 family I enzymes, and successfully discovered the selective P450 1A1 inhibitors 4'-ethynylflavone and 6-ethynylflavone, and selective P450 1A2 inhibitors 7,8-furanoflavone and 7,8-pyrano-4-trifluoromethylcoumarin. *In cell* P450 1A2 inhibition assays show that 7,8-pyrano-4-trifluoromethylcoumarin decreases the MROD activity in HepG2 cells at concentrations higher than 1 μM, suggesting the possibility of developing P450 1A2 inhibitors as cancer preventive agents.

Wiese/Burow Subproject

In year one, we developed the methods we use to perform virtual screening of the phytochemical and marketed drug databases. This involved obtaining all crystal structures of the ERalpha ligand binding domain, sorting these structures by ligand type and structure characteristics, and then comparing and optimizing ligand receptor docking protocols. At the same time, we obtained the phytochemical and marketed drug databases, and started the process of filtering for compounds with potential to bind ER that will go into the virtual screening process. Two pharmacy students were trained and then involved in the molecular modeling as well as trained for the *in vitro* validation phase of the Project.

In Year 2, we developed a better way to sort and screen the two molecule databases based on the similarity of the database members to the bound ligands in all available ER LBD crystal structures. To carry out this method, training was obtained and a resorting of the databases is underway. Ligand-receptor docking methods have been evaluated and a standard protocol showing high performance in ER LBDs has been established. One pharmacy student and a new research assistant were trained and are started generating bioassay data. A test set of 29 natural product stilbenes has been obtained and characterized for ER agonist and antagonist activity in a sensitive reporter gene system.

In Year 3 we have completed the dose response characterization of the 8 stilbenes shown to be active in Year 2. This data was presented in a poster at the ACS meeting in Spring 2014. Modeling analysis of these compounds presents ER binding modes consistent with the activity shown. We have identified 5 stilbenes with estrogen agonist activity and 3 stilbenes with antiestrogen activity. Of these antiestrogens 2 may bind ER in the typical direct antagonist mode while one may bind as in indirect antagonist.

In Year 4 we have completed modeling simulations of the antagonist activity of stilbenes 4, 9 and 28 as well as the simulations of the agonist activity of stilbenes 10,11,15, 33 and 34. A manuscript describing the *in vitro* activity and modeling of these compounds

in is preparation. A novel pharmacophore and docking method has been developed and applied to searching the National Pharmaceutical Collection database resulting in 17 pharmaceutical chemicals that are not known for estrogen activity, with potential for estrogen activity.

Sridhar/Jones/Stevens Subproject

Over the years 1 and 2, we were able to identify molecules that target two kinases, namely, PIM1 and CK1d, which play important roles in prostate cancer and breast cancer. Several compounds were found that inhibited MCF7 breast cancer cell line and HER2Δ16. Development of these lead compounds using molecular modeling and organic synthesis will give us potential drug candidates for breast cancer and prostate cancer. The dose response curve studies are ongoing for these two kinases. Based on the results further modification of the lead molecules will be attempted towards the goal of achieving better potency and selectivity for these two kinases. In the meantime, new database searches will be initiated based on the docking studies of known kinase inhibitors on HER2 to identify new core structures as lead molecules with the final goal of finding a new drug candidate for breast cancer.

In year 3 we have performed kinase inhibition assays and toxicology assays for the compounds that showed good inhibition of MCF7/HER2 and MCF7/ HER2Δ16 cell lines. Based on the bioactivity data and toxicity data, we have narrowed down the minimum structural features of the lead quinone moieties. We have synthesized several new derivatives of the lead quinone molecules as potential drug candidates. These will be subjected to inhibition assays and toxicology studies. Additionally, we have found a new lead molecule 1H-indazol-3-ol (indazolone) through computational studies. Two derivatives of the 1H-indazol-3-ol molecules have been synthesized. Further synthetic work is in progress.

In year 4 we synthesized several new derivatives of the lead naphthoquinone core structure. These synthesized compounds were tested for growth inhibition of the MCF7/HER2 and MCF7/ HER2Δ16 cell lines. Four compounds were shown to exhibit better potency than lapatinib. The best compound RLS77 was then tested against several breast cancer cell lines that were trastuzumab sensitive (SKBR3 and BT474), trastuzumab resistant (SUM190PT and SUM225CWN) and triple negative (BT20, MDA-MB-468 and MDA-MB-231) and this compounds showed excellent inhibition of all of these cell lines. The IACUC application for *in-vivo* xenograft studies has been approved.

Program

In addition to the significant amount of scientific research performed and data collected during the first, second, and third years of the Project, it is important to note the valuable

partnership developed between the two institutions involved. The productive collaboration formed between the Xavier University and Tulane Cancer Center researchers participating in this program, once again proves the value and importance of inter-institutional research/training projects. The different training activities and the number of trainees involved in the various aspects of the subprojects also positively impact the future cancer research environment in the area. This breast cancer research project is still in its early stages and is expected to develop significantly over the next years.

REFERENCES:

Foroozesh/Beckman/Burow Subproject

1. J. W. Antoon, J. Liu, M. M. Gestaut, M. E. Burow, B. S. Beckman, and M. Foroozesh, *J Med Chem*, **2009,** 52, 5748-5752.

2. J. Chun, G. Li, H. S. Byun, and R. Bittman, *J Org Chem*, **2002**, 67, 2600-2605.

3. J. Liu, J. W. Antoon, A. Ponnapakkam, B. S. Beckman, and M. Foroozesh, *Bioorg Med Chem*, **2010**, 18, 5316-5322.

4. A. P. Struckhoff, R. Bittman, M. E. Burow, S. Clejan, S. Elliott, T. Hammond, Y. Tang, and B. S. Beckman, *J Pharmacol Exp Ther*, **2004**, 309, 523-532.

5. Azuma, H; Ijichi, S; Kataoka, M. Short-chain 3-ketoceramides, strong apoptosis inducers against human leukemia HL-60 cells. *Bioorg Med Chem* **2007**, 15, 2860-7.

6. Wong, L.; Tan, S. S.; Lam, Y.; Melendez, A. J. Synthesis and evaluation of sphingosine analogues as inhibitors of sphingosine kinases. *J Med Chem* **2009**, 52, 3618-26.

Wiese/Burow Subproject

NA

Sridhar/Jones/Stevens Subproject

1. F. Castiglioni, E. Tagliabue, M. Campiglio, S.M. Pupa, A. Balsari, and S. Menard, *Endocr Relat Cancer*, **2006,** 13, 221-32.

2. H.T. Cuypers, G. Selten, W. Quint, M. Zijlstra, E.R. Maandag, W. Boelens, P. van Wezenbeek, C. Melief, and A. Berns, *Cell,* **1984,** 37, 141-50.

3. S.M. Dhanasekaran, T.R. Barrette, D. Ghosh, R. Shah, S. Varambally, K. Kurachi, K.J. Pienta, M.A. Rubin, and A.M. Chinnaiyan, *Nature,* **2001,** 412, 822-6.

4. G. Giamas, L. Castellano, Q. Feng, U. Knippschild, J. Jacob, R.S. Thomas, R.C. Coombes, C.L. Smith, L.R. Jiao, and J. Stebbing, *Nucleic Acids Res,* **2009,** 37, 3110-23.